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Vitamin E Deficiency in the Rat

V. UTERINE CHANGES IN CHRONIC DEFICIENCY

By M. M. O. BARRIE SWEETEN, *Department of Pharmacology, University of Toronto, Canada*

(*Received* 28 *July* 1943)

In a previous paper [Barrie, 1938*b*] I described the degenerative pigmentation of the uterus produced in the rat by chronic vitamin E deficiency, which was first observed by Martin & Moore [1936, 1938]. It seemed important to find out whether or not this condition could be cured, at any stage of its development, by administration of vitamin E, and biopsies of the uterus at various stages before and during treatment have therefore been carried out.

EXPERIMENTAL

Laparotomy was performed under ether anaesthesia and a small portion of the left horn of the uterus was removed. This entailed removal of the left ovary. In one case, rat 1081, the ovary was implanted in the peritoneum. The whole operation was carried out under strictly aseptic conditions, the wound being kept as small as possible.

Twenty-nine vitamin E-deficient rats were chosen, all of which had received from the time of weaning the same vitamin E-free diet, E5 [Barrie, 1938*a*], but for varying periods ranging up to 10 months. The animals received daily doses of a vitamin E concentrate or synthetic *dl*-α-tocopherol for a period of approximately 2 weeks following operation. In 18 of the rats the procedure was then repeated, the old wound being reopened and another piece of the uterus being removed; a third operation was performed on two of these animals. There was no sepsis and no deaths in any of the 49 operations.

After further treatment with vitamin E, the animals were mated and then killed either at the end of the resorption, or after the litter had been weaned. The last dose of vitamin E was always given on the day of mating.

One of the operated rats, 1081, did not become pregnant in spite of five matings with different males when it was on full oestrus. The animal was killed and a post-mortem examination was made, but no abnormality other than the uterine discoloration was found. Another animal, 1541, was exceptional in that it never came on oestrus following operation; anoestrous smears were obtained on 73 consecutive days, after which it was killed and post-mortem examination revealed a cyst on the remaining ovary.

The size and colour of the pieces of uterus removed at operation were carefully noted at the time of removal; they were then fixed in Kaiserling's fluid. The colour was restored by treatment with 1% (w/v) sodium hydrosulphite in *N*/1 NaOH, after which the pieces were washed with water and transferred to glycerine. In this way the pieces of uterus removed at different stages could be compared directly as well as by comparison of the notes made at the operations. It was found that the colour of the uterus varied from the normal pinkish white to deep chocolate brown, and a scheme of assessment of the degree of pigmentation was drawn up as follows:

Colour	Rating
Normal (pinkish white)	0
Yellow	1
Dark yellow or orange	2
Orange brown	3
Red-brown or brown	4
Chocolate brown	5

Most of the animals received a small dose of synthetic *dl*-α-tocopherol and a larger dose of vitamin E concentrate, 1 ml. of the concentrate being equivalent to 8·75 mg. of the *dl*-α-tocopherol when measured by the amount required to maintain pregnancy in vitamin E-free rats. All the doses were given on consecutive days (omitting Sundays) in the period between operations. The concentrate was given undiluted in doses of 0·25 or 0·5 ml., and the *dl*-α-tocopherol as a solution in sesame oil, 10 mg./ml., the daily dose being 0·25 ml.

RESULTS

Tables 1 and 2 summarize the case histories of all the rats, the animals being arranged in groups according to age. The day of the first biopsy is called day 1, and all other stages of the experiment are given in days from this point.

The doses of vitamin E given are expressed in terms of *dl*-α-tocopherol and the amount received between operations is given in addition to the total dose. Where there were several matings and no pregnancy ensued, the number of matings is recorded, but not the actual days of mating. In these cases the final examination was delayed as it was not considered desirable to kill the animals until it became certain that they were infertile. Where there was more than one mating before implantation took place, the day of the last mating is recorded, followed by the number of matings in brackets.

Table 3 summarizes Tables 1 and 2. The animals are again grouped according to age, but the average rating of each of these age groups has been calculated, first at the beginning of the experiment and then after treatment with vitamin E. The results of treatment are divided into those in which treatment was followed by a successful pregnancy and those in which it was followed by resorption or birth of a dead litter. These groups are of necessity very small as they become subdivided according to response; for this reason, no statistical interpretation has been attempted. The figures give a clear indication that treatment with vitamin E alone has

Table 1. *The individual uterine condition, spacing of operations and time of mating, and the gestational history of vitamin E-deficient female rats before, during and after treatment with vitamin E*

Rat no.	Uterine condition			Time of operation (day)	Time of mating (day)	Result of mating	Uterine condition post-mortem
	Op. 1	Op. 2	Op. 3				
1137	5	4	—	1 26	48	Resorption	3
1216	5	5	5	1 21 34	46	„	1
1241	5	4	—	1 23	42	Litter 1 (still-born)	5
1091	3	4	—	1 25	47	Resorption	3
1541	3	—	—	1	—	—	0
1081	3	3	—	1 25	—	—	5
1079	4	3	—	1 25	48	Resorption	1
1482	4	—	—	1	20 (5 mates)	Litter 1	3
1542	4	—	—	1	20	Resorption	3
1448	4	4	—	1 25	51	Litter 3 (2 still-born)	0
1540	4	—	—	1	20	„ 5	0
1484	4	—	—	1	30	Resorption	3
1904	5	—	—	1	20 (2 mates)	Litter 7	1
1537	4	4	—	1 24	41	„ 2	0
1707	4	1	—	1 25	49	„ 7	0
1539	2	3	—	1 23	40	„ 3	1
1538	3	3	—	1 23	40	„ 4	2
1671	3	3	—	1 13	29 (2 mates)	Resorption	4
1668	2	2	3	1 19 34	66 (5 mates)	Litter 5	1
1708	0	0	—	1 14	30	Resorption	0
1917	5	—	—	1	19 (4 mates)	Litter 6	0
1903	4	—	—	1	57 (2 mates)	„ 5	0
1715	4	4	—	1 23	42	„ 6	1
1954	2	—	—	1	15	Resorption	3
1961	2	—	—	1	15 (3 mates)	Litter 4	0
1844	2	2	—	1 25	55	„ 7	1
1837	4	4	—	1 13	25	„ 7 (1 still-born)	0
1831	2	2	—	1 13	25 (2 mates)	„ 3	3
2081	0	—	—	1	29 (3 mates)	„ 6	0

Table 2. *The distribution and total doses of vitamin E given to vitamin E-deficient female rats (in terms of dl-α-tocopherol), and the period on a vitamin E-free diet before operation*

Rat no.	Months on diet	Vitamin E given (as mg. *dl*-α-tocopherol)					
		As concentrate			As tocopherol on day of mating	Total dose	
		Between Op. 1 and Op. 2	Between Op. 2 and Op. 3	Between last Op. and mating		As concentrate	As *dl*-α-tocopherol
1137	10	35	—	40	5	75	5
1216	10	96	15*	0	5	96	20
1241	9	88	—	0	5	88	5
1091	8	35	—	40	5	75	5
1541	8	—	—	61	—	61	—
1081	8	70	—	0	—	70	—
1079	8	35	—	40	5	75	5
1482	8	—	—	66	5	66	5
1542	8	—	—	61	5	61	5
1448	8	35	—	40	5	75	5
1540	8	—	—	61	5	61	5
1484	8	—	—	61	5	61	5
1904	7	—	—	48	5	48	5
1537	7	—	—	83	5	83	5
1707	7	35	—	40	5	75	5
1539	7	83	—	0	5	83	5
1538	7	83	—	0	5	83	5
1671	7	35	—	57	5	92	5
1668	6	10*	96	0	5	96	15
1708	6	92	—	0	5	92	5
1917	6	—	—	48	5	48	5
1903	6	—	—	48	5	48	5
1715	6	88	—	0	5	88	5
1954	6	—	—	48	5	48	5
1961	6	—	—	105	5	105	5
1844	5	44	—	30	5	74	5
1837	5	92	—	0	5	92	5
1831	5	92	—	0	5	92	5
2081	4	—	—	83	5	83	5

* These doses were *dl*-α-tocopherol.

Table 3. *The average effect of treatment with vitamin E on the uterine colour rating of vitamin E-deficient rats treated after varying periods on a vitamin E-free diet*

Months on diet	Average rating	No. of rats	Average rating after treatment	No. of rats	Average rating after pregnancy	No. of rats	Average rating after resorption	No. of rats
4	0·0	1	—	—	0·0	1	—	—
5	2·7	3	2·7	3	1·3	3	—	—
6	2·7	7	2·2	3	0·4	5	1·5	2
7	3·5	6	2·8	5	0·8	5	4·0	1
8	3·7	9	3·5	4	1·0	3	2·5	4
9	5·0	1	4·0	1	—	—	5·0	1
10	5·0	2	4·5	2	—	—	2·0	2
Overall averages	3·3	29	3·1	18	0·75	17	2·6	10

very little effect on the uterine discoloration, the rating being reduced from 3·3 to 3·1, a reduction which obviously has no significance, whereas the same treatment followed by pregnancy and the birth of a living litter reduces the rating to 0·75, which must be considered significant, even on the small amount of available data. On the other hand, treatment with vitamin E followed by a gestation

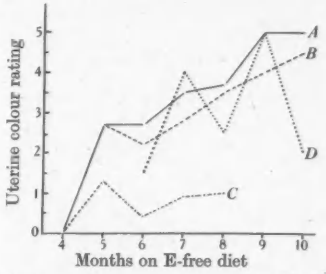

Fig. 1. Uterine colour rating before and after treatment with vitamin E. Curve *A*, before treatment. Curve *B*, after treatment. Curve *C*, after treatment and successful gestation. Curve *D*, after treatment and resorption, or unsuccessful gestation.

in which resorption occurred reduces the rating only to 2·6. In each treated group an improvement was obtained; this was only 0·2 after vitamin E treatment, 0·7 after vitamin E treatment and resorption, but 2·5 after vitamin E treatment and a successful gestation. A similar comparison can be obtained by considering the different age groups separately, but owing to the small number of animals, it would seem better to consider the averages of these groups. Fig. 1 clarifies this point by expressing the result graphically.

DISCUSSION

It appears clear from these results that the uterine discoloration produced by chronic vitamin E deficiency is a reversible condition, but that administration of vitamin E alone, even if given in massive doses, will have very little effect unless the animal is mated and gestation ensues. If the gestation is normal and a live litter results there is a marked, and in many cases, complete recovery from the pigmented condition. If a resorption gestation occurs the improvement is much less. It seems probable that pregnancy increases the circulation in the uterine muscle and is therefore the chief factor in the removal of the pigment.

Unfortunately, it is impossible to control the experiment by producing pregnancy and giving no vitamin E, as sterility is well established before the onset of pigmentation. It can only be shown that vitamin E alone is ineffective.

These results agree very well with those of Martin & Moore [1939], who found very similar uterine discolorations in their vitamin E-deficient rats. Unfortunately they did not examine the uteri before and after treatment, so that their results cannot be conclusive. These authors also say that after parturition the uterus of such rats remains abnormal, but since there was no previous examination, there is nothing to show that improvement has not taken place, and the foregoing experiments indicate that this was probably the case.

SUMMARY

1. Chronic vitamin E deficiency in the female rat produces uterine discoloration.

2. This discoloration is not curable by vitamin E administration alone, but yields to this treatment if accompanied by successful pregnancy.

3. It is suggested that the increased circulation to the uterine muscle during pregnancy is responsible for the removal of the pigment.

I wish to thank the Directors of the British Drug Houses Ltd., for permission to publish this work which was carried out while I was working in their laboratories.

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The Estimation of Creatine and of Diacetyl

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(*Received* 10 *June* 1943)

Voges & Proskauer [1898] observed that when strong alkali was added to broth cultures of certain species of bacteria there developed, after an interval of time, a pink colour. Subsequently the work of Harden [1906] and of Harden & Norris [1911] established that the chemical reaction responsible was one between diacetyl and creatine (or certain similar substances). Attempts to make the reaction quantitative [Walpole, 1911; Eggleton & Eggleton, 1928] were only partially successful. The reaction was further studied by Dulière [1929], Lang [1932] and Müller [1935], but it was the discovery by Barritt [1936], that α-naphthol greatly intensifies the colour, which made it possible for the first time to use this reaction as a means of estimating either creatine or diacetyl. The present paper presents some additional facts about the reaction and methods of estimation based on it.

EXPERIMENTAL

Preliminary experiments

Preliminary experiments confirmed Barritt's observations and also demonstrated that the reaction took place rapidly at room temp. Early in the research it was noticed that colour development invariably began at the surface of the liquid, suggesting that oxygen was involved in the reaction. This was proved by studying the reaction in the absence of O_2, and by measuring the amount of O_2 used. When the reagents were mixed under an atmosphere of O_2-free hydrogen, no colour formation occurred even after a period of 4 hr., whereas the control in air showed maximum colour development within 30 min. After 4 hr. exposure to O_2-free H_2, with no pigment production, air was admitted and immediately colour formation began and reached approximately the same intensity as the control.

The Warburg manometric apparatus was employed to measure the O_2 used during the reaction. Two ml. of the stock alkali (see later) or 2 ml. of 1% α-naphthol in the stock alkali, were placed in the main chamber of the manometer cup, and the creatine and the diacetyl solutions were placed in the side bulb. By this means the following points were established (for a temp. of 26°): (1) Creatine in alkaline solution does not absorb O_2. (2) α-Naphthol in alkaline solution consumes O_2 at a slow but steady rate over a period of many hours. (3) Diacetyl in alkaline solution shows a rapid initial O_2 uptake which falls off, sometimes completely, after 30 min. It amounts to about 1 mol. O_2/mol. diacetyl. (4) On mixing diacetyl with α-naphthol in alkali there is a consumption of O_2, the rate of which is approximately equal to the sum of the rates of O_2 consumption of diacetyl and α-naphthol separately. (5) The addition of diacetyl and creatine to the alkali causes a rapid consumption of O_2 for the first 30 min., which falls off to a low steady rate. The presence of excess of creatine increases by 2- or 3-fold the amount of O_2 consumed by diacetyl during this 30 min. In other words, O_2 is involved in the Voges-Proskauer reaction, though it is not possible to say how much, since under the conditions of these experiments some of the diacetyl must have oxidized without reacting with creatine. (6) When diacetyl and creatine are added to α-naphthol in alkaline solution, there is an initial rapid phase of O_2 consumption lasting about 30 min., followed by a slow steady O_2 uptake lasting over a period of hours. The amount of O_2 used by this mixture is greater than when creatine is omitted, indicating that the modified Voges-Proskauer reaction also involves an oxidation by molecular O_2. The results of a typical experiment are expressed graphically in Fig. 1. In this experiment, the amount of

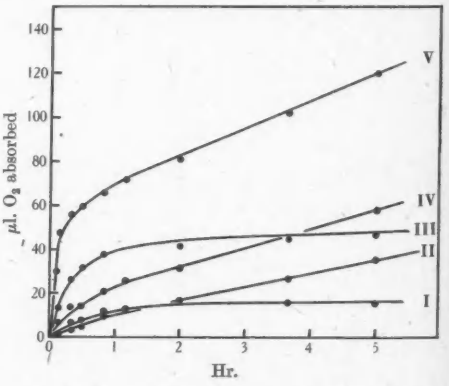

Fig. 1. O_2 uptake at 26° (measured in Warburg manometers) of the following mixtures dissolved in 2 ml. of stock alkali.

I.	α-naphthol	0 mg.,	diacetyl	0·2 mg.,	creatine	0	mg.
II.	,,	10	,,	,,	0·0	,,	,,	0	,,
III.	,,	0	,,	,,	0·2	,,	,,	7·5	,,
IV.	,,	10	,,	,,	0·2	,,	,,	0	,,
V.	,,	10	,,	,,	0·2	,,	,,	7·5	,,

diacetyl (0·2 mg.) was the limiting factor, creatine and α-naphthol being present in excess, 7·5 and 10 mg. respectively. (7) The rate of O_2 consumption is approximately equal to the rate of colour development. The rate of O_2 consumption was obtained as the difference between the rate of O_2 consumption of a mixture of diacetyl, creatine and

α-naphthol and the rate of O_2 consumption of a similar mixture of diacetyl and α-naphthol. Colour development was measured by means of the Zeiss Pulfrich photometer. Within the limits of experimental error, which in this case are large, owing to the speed of the reaction and the presence of side reactions, colour development and O_2 consumption ran parallel.

These experiments show that the Voges-Proskauer reaction and the Barritt reaction both involve an oxidation by molecular O_2, and in consequence special attention must be paid to this fact when using the reaction for the estimation of either creatine or diacetyl.

Nature of the pigment formed in the Voges-Proskauer and Barritt reactions

The isolation of the pigment formed in the modified reaction has been attempted, but so far it has proved impossible to obtain it in the crystalline state. The impure product has some interesting properties. In the solid state it has been stable for 4 years. In alkaline solutions of pH 10 it is coloured red; at pH 8 it is purple, and at pH 6 it is brown; it is least soluble at pH 4. These facts suggest that it is a compound with three ionizing groups and an isoelectric point at pH 4. In concentrated HCl or H_2SO_4 the pigment is quite stable and coloured purple. In solution at pH 12, 40 mg. impart the same intensity of colour as 7 mg. of creatine in excess of diacetyl and α-naphthol, or 5 mg. of diacetyl in excess of creatine and α-naphthol.

The pigmented solution produced in the presence of α-naphthol was examined spectroscopically (our thanks are due to Dr L. E. Bayliss for valuable advice and help) and the absorption spectrum was found to have a single peak centred on wave-length 5250 A., the extinction coefficient (E) having half the peak value at 4650 and 5660 A.

The Zeiss S50 and S53 filters proved to be suitable for the photometric measurement of the pigment. The question of the optimum conditions for the development of maximum colour from small amounts of creatine and small amounts of diacetyl was then investigated.

Investigation of reaction conditions

Alkali. A solution of NaOH was used both in the original Voges-Proskauer test and in the Barritt modification. Investigation of the degree of alkalinity necessary for maximal colour development in the Barritt reaction showed that (*a*) no colour developed when the solution was made alkaline with $NaHCO_3$, (*b*) pigment formation occurred when either Na_2CO_3 or NaOH was used, and (*c*) maximum colour development occurred when a mixture of NaOH and Na_2CO_3 was used. The concentration of these compounds was not critical. A stock alkali solution was made up containing 30 g. NaOH and 80 g. Na_2CO_3 (equimolar amounts) in distilled H_2O and made up to 500 ml. Two ml. of this solution were used for the estimation.

α-Naphthol. Barritt used a solution of α-naphthol in ethanol, but as this rapidly becomes pigmented on standing, a solution in the stock alkali was used instead. The amounts of α-naphthol required for maximal colour development proved to be surprisingly large, about 500 mol./mol. of creatine, and at first the activity of the α-naphthol was thought to be due to the presence of some impurity. However, after thrice distilling in steam from an acid solution, the α-naphthol was neither more nor less active than before. Steam distillation yielded a perfectly white, crystalline product, which, if kept in a dark bottle, did not become discoloured. All subsequent samples of α-naphthol were purified in this manner. The solution of α-naphthol in alkali does not keep more than 2 or 3 hr. and in consequence it was always made up just before it was required. A 1% solution was used.

Diacetyl. A stock solution is prepared from dimethyl glyoxime (British Drug Houses Ltd., 'Analytical Reagent') by heating 1·6 g. with 200 ml. of 5N H_2SO_4 in an all-glass distilling apparatus and collecting the first 50 ml. of distillate; this is made up to 100 ml. with H_2O. The amount of dimethylglyoxime taken yields about 1 g. diacetyl (as assayed by the van Niel [1927] method). This yield (85% of theoretical) is fairly constant, and the solution is stable for at least a month if kept in the ice-box.

Excess diacetyl causes production of a yellow pigment which tends to obscure the colour produced by the creatine; a deficiency causes subnormal colour production. For amounts of creatine up to 60 μg. in a final volume of 10 ml., 1 ml. of 1 : 20 dilution of the stock 1% solution was found to be optimal.

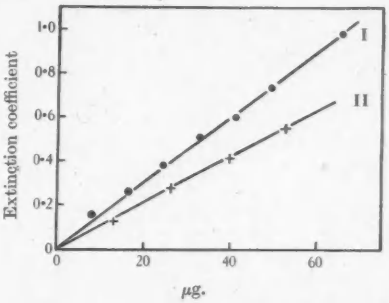

Fig. 2. Colour intensity (measured as extinction coefficient, S53 filter, 10 mm. cuvettes) produced by different amounts of creatine (II) and diacetyl (I) when treated in the manner described in the text.

Creatine. The sample used (British Drug Houses Ltd.) was recrystallized from distilled H_2O. With 0–50 μg. of creatine the colour was proportional to the amount of creatine present. Similarly, it was shown that when creatine was in excess the colour was proportional to the amount of diacetyl present. The results (Fig. 2) show that there is a linear relationship between intensity of colour and amount of diacetyl, and that the chromogenic powers/μ.mol. of diacetyl and creatine are about equal. This may indicate that the pigment contains diacetyl and creatine in equimolar proportions.

METHODS OF ESTIMATION

(*a*) *Creatine*

Reagents. (1) Stock alkali. (2) Stock 1% diacetyl diluted 1 : 20 before use. (3) α-Naphthol purified by steam distillation. A 1% solution in the stock alkali is made up just before use. (4) Saturated solution of creatine.

Procedure. To a neutral solution of creatine (containing not more than 60 μg.) in a standard

10 ml. flask are added 2 ml. of the 1% α-naphthol in alkali followed by 1 ml. of the diluted diacetyl. The solution is briskly shaken and made up to the mark with distilled H_2O and left for 30 min. The colour may be measured with the Zeiss Pulfrich photometer (S 53 filter and a reagent blank in the control cuvette), or by direct colorimetry with standard creatine solutions prepared in the same way. There is no interfering colour from the reagents alone and the intensity of the colour is stable for at least 2 hr. The extinction coefficient for creatine under these conditions is 0·0105/μg. (10 mm. cuvette, and S 53 filter).

Many guanido compounds react under these conditions [cf. Barritt, 1936]. Of those that we examined quantitatively, arginine, guanidine and glycocyamine produce about one-ninth of the colour given by an equivalent amount of creatine. Creatinine and freshly prepared creatine phosphate do not react under these conditions.

(*b*) *Diacetyl*

Reagents. As for the estimation of creatine (see p. 527).

Procedure. To a solution of diacetyl (containing not more than 100 μg.), in a standard 15 ml. flask, are added 4 ml. of a solution made up from 1 part saturated aqueous creatine and 3 parts 1% α-naphthol in stock alkali. The flask is briskly shaken, made up to the mark with distilled H_2O and allowed to stand for 30 min. It is then estimated in the photometer with a reagent blank in the control cuvette, or by direct vision colorimetry. Of the two the latter method is to be preferred owing to the presence of a slight variable blank error which it has proved impossible to eradicate.

Diacetyl does not appear to be formed by cells to any large extent; its reduction product, acetyl methyl carbinol, is far more common. This substance is readily oxidized to diacetyl by refluxing with ferric chloride [van Niel, 1927] and the diacetyl so formed may be removed by distillation, e.g. in the micro-Kjeldahl apparatus provided by Quickfit and Quartz Ltd. The following experiment illustrates the speed with which this occurs. 1 ml. of diacetyl solution containing 163 μg. diacetyl was pipetted into each of four distillation flasks and distilled in turn for varying periods of time. The distillate and washings were collected in calibrated measuring cylinders, the volume noted and 5 ml. taken in each case for analysis (Table 1).

Table 1. *Recovery of diacetyl by distillation*

Exp. no.	1	2	3	4
Diacetyl added (μg.)	163	163	163	163
Time of distillation (min.)	5	10	15	20
Vol. of distillate washings (ml.)	8·8	11·4	15·4	22
Diacetyl recovered (μg.)	164	162	154	159

In Exp. 1, 5 ml. of distillate were collected, the remainder coming from washings. It appears therefore that by collecting 10 ml. of distillate a quantitative recovery is assured.

Sources of interference

(1) The use of $CCl_3.COOH$ as a protein precipitant can cause some interference, since it reacts with alkaline α-naphthol solutions, producing an intense blue pigment which entirely masks the colour given by creatine. This reaction occurs only in ultra-violet light. If the colour development is carried out in the dark room or in the light of a tungsten filament lamp, $CCl_3.COOH$ does not interfere.

(2) Urine, and $CCl_3.COOH$ extracts of liver, contain substances which inhibit the reaction between diacetyl, creatine and α-naphthol. (The reaction between creatine and diacetyl is similarly inhibited, and Walpole believed that NH_3 was responsible for the inhibition in the case of urine.) For example, the colour production from 30 μg. creatine in excess diacetyl was reduced to 74% by the presence of 1 ml. of a normal urine, the effect being proportional to the amount of urine added. Similarly the colour intensity due to diacetyl in the presence of excess creatine was reduced to 68% of normal by 1 ml. of the same urine. This marked inhibitory action was not shown by urea, NH_3, uric acid or creatinine when added in amounts likely to be present in 2 ml. of urine. Glycine, however, proved to be an inhibitor. The colour intensity with 30 μg. of creatine was reduced by one-half in the presence of 4 mg. of glycine. Alanine and glutamic acid did not show this property, but β-alanine and β-alanyl histidine (carnosine) were inhibitory, being about 60% as active, on a molar basis, as glycine.

These substances also inhibited the colour production by diacetyl in the presence of excess creatine, indicating that the mechanism of the interference is not a destruction of either diacetyl or creatine, but a reaction with some compound formed intermediately in the Barritt reaction.

Discussion of methods

It appears from the above experiments that omega amino-acids may be responsible for the inhibitory action of liver extracts and urine. No ornithine or lysine was available so it was impossible to test the hypothesis that all omega amino-acids inhibit the reaction, nor was the effect of primary amines tried, though it may well be that the inhibition is due to the terminal amino group $—CH_2.NH_2$. In the light of these experiments, the claim of Lang [1932] to be able to estimate arginine in protein digests by a diacetyl method seems to require confirmation. Indeed, we observed no colour in a neutralized acid hydrolyzate of gelatine which had been treated with excess diacetyl and α-naphthol in alkaline solution, despite the presence of large quantities of arginine.

The use of the method for the estimation of arginine or creatine is therefore limited by these inhibitors. However, most animal tissues, with the

exception of liver, appear to contain insignificant quantities of interfering substances. In the case of skeletal muscle with its relatively high content of β-alanine in the form of carnosine and anserine, the situation is mitigated by the fact that the creatine content is also very high. Thus, only 0·1 ml. of a 1 : 10 CCl_3.COOH extract of skeletal muscle is required for the estimation of free creatine, and in this small volume the concentration of β-alanine-containing dipeptides is negligible. None the less, if the method is used in connection with muscle creatine, the possibility of interference from these sources should be borne in mind. It is possible that a preliminary separation of the creatine by selective adsorption would overcome the difficulties. In the estimation of diacetyl the preliminary distillation removes these sources of interference.

RESULTS

Except in the case of liver, no noticeable abnormalities have been encountered with animal tissues. Table 2 gives figures obtained with neutralized 1 : 10 CCl_3.COOH extracts of certain tissues of the rat. The animals were killed by stunning.

Table 2. *Creatine content of animal tissues*

Tissue	Present method—rat (mg./100 g.*)	Picrate method [Hunter, 1928] (mg./100 g.*)
Skeletal muscle	524	470—
Cardiac muscle (ventricle)	192	168–223 (rabbit)
Smooth muscle (small intestine)	30·4	27·1 (rabbit)
Whole brain	132	114 (rabbit)
Lung	8·1	No figures
Spleen	6·5	19·1
Kidney	19·9	15·8–29 (dog)
Testis	267	212 (sheep)
Liver	0	23·3 (rabbit)

* Wet weight.

A portion of the tissue was removed, freed from blood by washing with distilled H_2O, dried on filter paper, weighed, and ground with 10 times its weight of 4 % CCl_3.COOH and a little sand. The insoluble material was filtered off and portions of the clear filtrate neutralized before development of the colour. It is assumed that the pigment formed was entirely derived from creatine. For comparison are given figures taken from Hunter [1928]; where no figures for the creatine content of rat tissues were available, those for corresponding tissues of other species are quoted. Hunter's results were all obtained by converting the extracted creatine into creatinine and determining the creatinine by the Jaffé reaction.

SUMMARY

1. Barritt's observation, that the Voges-Proskauer colour test for diacetyl is greatly increased in sensitivity in the presence of α-naphthol, is confirmed.

2. Barritt's reaction can also be used as a sensitive test for creatine.

3. Both the Voges-Proskauer and the Barritt reactions involve an oxidation by molecular oxygen.

4. Certain amino-acids interfere with the colour production in the Barritt reaction when the latter is used to estimate either creatine or diacetyl.

5. A similar interference is noted when urine and extracts of liver are present in the reaction mixture.

6. Arginine, guanidine, and glycocyamine react in a similar manner to creatine, but produce less colour. Creatinine and creatinephosphoric acid produce no colour.

7. Methods are described for the estimation of creatine and of diacetyl, in quantities up to 50 μg.

8. A reaction between α-naphthol and trichloroacetic acid is described, which occurs only under ultra-violet excitation.

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Vitamin Methods

7. A NEW MODIFICATION OF THE *p*-AMINOACETOPHENONE METHOD FOR ESTIMATING NICOTINIC ACID IN URINE

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Harris & Raymond [1939] developed a method for estimating nicotinic acid in urine with the object of assessing the nutritional status in this vitamin, along the same lines as those employed in the saturation tests previously devised for vitamin C [Harris & Ray, 1935] and vitamin B_1 [Harris & Leong, 1936]. The method was based on the cyanogen bromide reaction of König [1904], a novel feature being the reagent used as the aromatic amine, namely, *p*-aminoacetophenone. Although other aromatic amines have also been used for determining nicotinic acid, e.g. aniline and *p*-aminophenol, several investigators have concluded that Harris & Raymond's reagent is the most advantageous [Naganna, Giri & Venkatesam, 1941; Stotz, 1941; Arnold, Schreiffler & Lipsius, 1941].

With urines and highly coloured extracts the colour reaction is always less easy to perform than with colourless material. To overcome this difficulty, several alternative procedures have been suggested, but some of these are too tedious, while others run the risk of loss of nicotinic acid. For this reason we have undertaken an extensive study with special reference to the following: the removal of interfering coloured and non-coloured substances; the problem of the 'blank' correction; the conditions of the preliminary conversion of related substances to nicotinic acid. The optimal conditions for the reaction of CNBr with nicotinic acid and the subsequent development of colour [Harris & Raymond, 1939; Kodicek, 1940 *a*, *b*] have been reinvestigated with regard to the complex composition of urinary extracts.

As a result, an improved modification of the *p*-aminoacetophenone method has been devised, which has the advantage of being simple and reliable. The method, which is based on the original procedure of Harris & Raymond [1939], has been used in this laboratory for over two years with good results.

GENERAL CONSIDERATIONS

(1) *Removal of interfering colour*

There are, in general, three methods of estimating a substance in a coloured solution: (*a*) by removing the substance from the solution, thus freeing it from the interfering colour; (*b*) by eliminating the colour from the solution without losing the compound to be determined; and (*c*) by estimating it in the coloured solution and allowing for the colour by a blank correction.

Separation of nicotinic acid from urine by removing it on adsorbents or by extracting it with an immiscible solvent is not very practicable owing to the difficulty of effecting a complete separation, and furthermore, some pigment is removed with the vitamin. A further disadvantage is the difficulty of eluting the vitamin quantitatively after adsorption. Perlzweig, Levy & Sarret [1940] claimed to have obtained good results by using Lloyd's reagent after preliminary oxidation. Dann & Handler [1941], using Perlzweig's decolorization procedure, confirmed this finding. The decolorization procedure, however, is more tedious than the oxidation method described in this paper and is much dependent upon various physical properties of the adsorbing agent. The adsorption of nicotinic acid by medicinal charcoal, as suggested by Giri & Naganna [1941], failed to give, in our hands, reproducible results. Swaminathan [1939] used charcoal in a different manner—to remove pigments from the urine, leaving nicotinic acid in the solution. He thereby eliminated the use of a blank correction except in cases in which the test solutions were still coloured. The adsorption was carried out in an alkaline medium, and it was claimed that no nicotinic acid was lost by this treatment. Melnick & Field [1940 *a*, *b*], like Swaminathan, used charcoal to remove interfering pigments, but performed the adsorption in acid alcoholic solutions. They admitted that their method was liable to give results with urine which are too high.

The method to be described combines principles (*b*) and (*c*) mentioned above, without recourse to adsorption. It consists of a preliminary washing of the hydrolyzed urinary solution with isobutanol, which removes part of the interfering coloured substances, and a subsequent oxidation of the remaining colour with potassium permanganate. The treatment usually produces a solution which is almost colourless. Any residual coloured or colourless interfering substances are allowed for by a special blank correction.

(2) *The blank correction*

Several ways of allowing for the blank correction have been suggested by various workers, but none of them seems to be entirely satisfactory. Bandier [1939*b*] used as a blank a sample of the urine treated as the 'unknown' but without the addition of the amine. The inadequacy of this blank, at least for *p*-aminoacetophenone, will be shown later when the different types of blank are compared. Harris & Raymond [1939] omitted CNBr from their blank, which had otherwise been treated exactly like the 'unknown'. This has been

criticized by Melnick & Field [1940*b*] on the ground that the amine reacts with the interfering constituents of urine to form a colour which is not produced in the presence of CNBr. We could not observe, however, any bleaching effect of CNBr under our experimental conditions, nor could we find in our new 'acid blank' any unspecific side reaction which would not be present also in the 'unknown' [cf. Melnick, Oser & Siegel, 1941]. Dann & Handler [1941] state that the cyanogen bromide procedure worked well only in a colourless solution. They adopt the procedure of Perlzweig *et al.* [1940] to produce an almost colourless extract, and introduce a blank correction which consists of the residual colour of the extract and the colour of the reagents measured separately. Although we agree with Dann & Handler that a colourless extract is to be preferred to a coloured one, we feel that their blank correction may show, with certain extracts, too low a value. Their blank is based on the assumption that a colourless extract contains no interfering substances which may react with the reagents to give a colour. We find that occasionally an apparently colourless solution may still react with the reagents to produce a slight colour. In the procedure to be described we have included all the reagents and the test solution in one blank carried out in such a way that the nicotinic acid in the test solution does not react.

(3) *Preliminary conversion to nicotinic acid of related substances*

Another point which has been the subject of much controversy among different workers is the question whether the conversion to nicotinic acid of related substances should be carried out in acid or in alkaline solution. Swaminathan digested the urine in 2 *N* NaOH for a period of 3 hr. Under such conditions, a considerable portion of trigonelline may be changed to nicotinic acid, which may account for his high values for nicotinic acid output. Melnick & Field, on the other hand, subjected the urine to acid hydrolysis. As pointed out by Perlzweig *et al.* [1940], this has the danger of preventing the complete conversion of nicotinuric acid to nicotinic acid. In the method of Harris & Raymond the digestion was carried out in *N* NaOH for 30 min. It will be shown later that this short digestion with the relatively dilute alkali is preferable, as it does not cause any appreciable conversion of trigonelline to nicotinic acid even in the presence of large quantities of the former compound. Moreover, it is much more convenient, and yields a lighter coloured solution than the method with acid digestion.

(4) *Optimal conditions for the König reaction and subsequent development of colour*

The various factors which influence the König reaction have been extensively investigated by Harris & Raymond [1939] and Kodicek [1940*a*]. There were, however, a few more points which needed further study, especially the conditions for the reaction of CNBr with nicotinic acid. All these factors have been investigated in the present work, and are discussed below in §§ IV and V.

EXPERIMENTAL

(I) *Comparison of preliminary acid and alkaline digestion*

Three 40 ml. portions of a sample of urine were digested with 20 ml. of 12 *N* HCl on a boiling water-bath for 5 hr., neutralized, and decolorized as described in § II(*a*) below. Further batches of eight 50 ml. portions of the same urine, four with added trigonelline and four without, were digested with *N* NaOH for 45 min. on the water-bath, neutralized and decolorized as before. They were then analyzed for nicotinic acid. It will be seen from Table 1 that the same values were obtained whether the digestion was carried out in acid or alkali, and, in the latter case, with or without the addition of trigonelline. The slight variations in the individual values are due to the experimental error, since, with the low values of extinction, the personal error in reading is from 5 to 10%.

Table 1. *Comparison of results with preliminary alkali and acid digestions*

Estimations of nicotinic acid in the presence and absence of trigonelline

	Digestion in alkaline solution				Digestion in acid solution.
	Extinction with				
Material	Acid blank	Urine	Urine + 10 μg. of nicotinic acid	Nicotinic acid found in urine (μg./ml.)	Nicotinic acid found in urine (μg./ml.)
Urine alone: Test 1	0·049	0·135	0·352	0·79	0·74
Test 2	0·052	0·141	0·355	0·83	0·73
Test 3	0·049	0·125	0·321	0·78	0·77
Test 4	0·049	0·146	0·350	0·95	—
Average	0·050	0·137	0·341	0·84	0·75
Urine, same specimen + 0·5 mg. of trigonelline/10 ml.	0·048	0·142	0·346	0·92	—
Urine, same specimen + 0·5 mg. of trigonelline/10 ml.	0·054	0·132	0·336	0·77	—
Average	0·051	0·137	0·341	0·84	—
Urine, same specimen + 1·0 mg. of trigonelline/10 ml.	0·053	0·157	0·381	0·93	—
Urine, same specimen + 1·0 mg. of trigonelline/10 ml.	0·055	0·146	0·360	0·85	—
Average	0·054	0·152	0·371	0·89	—

(II) *Decolorization of urinary digests*

After the preliminary digestion, which serves to convert related substances to nicotinic acid, the urine usually becomes quite dark in colour. It is necessary to remove the colour; otherwise the blank correction will be too high and the accuracy of the method much decreased. Since digestion with alkali always yields a lighter coloured solution than digestion with acid, and as shown above, trigonelline does not interfere, it was decided to use the former method of digestion and to carry out the decolorization of this solution. Two methods of decolorization were tried:

(*a*) *Charcoal adsorption.* The procedure used for decolorization with charcoal as recommended by Swaminathan [1938] was used with some modifications. The method is as follows:

After the preliminary digestion with alkali (unlike Swaminathan, we digested with *N* NaOH, i.e. 5 ml. of 40% (w/v) NaOH added to 50 ml. of urine, and the time of digestion restricted to 45 min.), the urine was neutralized with conc. HCl, and then adjusted to a less strongly alkaline reaction by the addition of 0·5 ml. of 40% NaOH. The precipitate was centrifuged off. The centrifugate was made up to 50 ml., and boiled with 1 g. of norite charcoal. The solution was filtered hot with suction and the charcoal on the filter paper boiled in about 30 ml. of dilute alkali (0·3 ml. of 40% NaOH to 100 ml. of water) for 1–2 min., filtered again and the residue washed with 20 ml. of the hot dilute alkali. The combined filtrates were brought to pH 6·5–6·8 with HCl and made up to 100 ml. The solution was then analyzed for nicotinic acid according to the procedure described below.

This procedure for decolorization gave good recoveries (Table 2). Larger amounts of charcoal (2 g. as used by Swaminathan), however, caused 20–40% loss of added nicotinic acid. The slight yellow colour that remained after adsorption was allowed for by a blank correction. However, owing to the difficulty of obtaining charcoal of uniform quality, this procedure was given up in favour of the second method to be described, which not only gives a more colourless extract but also has other advantages and gives more reliable results.

Table 2. *Recovery of added nicotinic acid after decolorization of urine with charcoal*

Exp. no.	Nicotinic acid in original urine (μg./ml.)	Nicotinic acid added (μg./ml.)	Additional nicotinic acid found (μg./ml.)	Recovery (%)
1	5·1	10·0	9·8	98
2	1·7	10·0	11·2	112
3	4·0	10·0	10·3	103
4	1·24	1·00	1·16	116
5	1·05	2·00	2·15	108
6	0·58	2·00	2·32	116
7	0·48	2·00	1·98	99
8	1·0	0·93	0·74	80
			Average	104

Standard deviation of single determination ±12.

(*b*) *Washing with isobutanol and oxidation with* $KMnO_4$. Urinary pigments may be effectively removed from solution by extracting them with immiscible solvents, such as phenol or benzylalcohol, but unfortunately a great proportion of nicotinic acid is removed as well. Isobutanol extracts, albeit incompletely, a considerable part of the urinary pigments, while it leaves practically all the nicotinic acid behind. The distribution coefficient of nicotinic acid between water-saturated isobutanol and isobutanol-saturated water below pH 4·5 is about 1 : 19. By using 1 vol. of isobutanol to every 2 vol. of urinary digest we have found it possible to remove a large part of the pigments without losing any significant amount of the nicotinic acid (Table 3), but for very accurate work, a correction factor could be introduced to account for the amount extracted by the isobutanol. The residual colour is then removed by oxidation with $KMnO_4$. The oxidation is carried out in acid solution at about 50°. A 4% solution of $KMnO_4$ is added drop by drop with continuous stirring until the colour of the permanganate stays for about 30 sec. The colour is allowed to disappear completely. This usually takes 5–10 min.* The solution is then neutralized carefully to pH 6·5–

Table 3. *Recovery of nicotinic acid after a solution in M/45 phosphate buffer* (pH 4·5) *had been washed with isobutanol*

	Extinction	Amount found (μg.)	Recovery (%)
(*a*) 10 μg. of nicotinic acid present:			
After washing with isobutanol	0·200	10·5	105
Control without treatment	0·251	10·4	104
(*b*) 25μg. of nicotinic acid present:			
After washing with isobutanol	0·786	23·8	95
After washing with isobutanol	0·809	24·2	97
Control without treatment	0·852	25·7	103

Table 4. *Recovery of added nicotinic acid with the new method*

	Nicotinic acid			
Exp. no.	In original urine (μg./ml.)	Added (μg./ml.)	Additional found (μg./ml.)	Recovery (%)
1	1·5	2·5	2·34	94
2	0·82	2·5	2·7	108
3	0·89	2·5	2·3	92
4	0·87	2·5	2·48	99
5	0·93	2·5	2·44	98
6	0·61	2·5	2·59	104
7	0·82	2·5	2·3	92
8	0·96	2·5	2·59	104
9	1·15	2·5	2·55	102
10	0·63	2·5	2·39	96
11	0·83	2·5	2·32	93
12	1·17	2·0	2·09	105
13	0·26	2·0	1·85	93
			Average	98·5

Standard deviation of single determination ±5·6.

* If the liquid is not sufficiently reducing, as with a solution of the pure vitamin, the excess of permanganate has to be reduced by the addition of a few drops of 0·5% H_2O_2. The excess of H_2O_2 must be removed (e.g. by catalase), as otherwise it may interfere with the subsequent development of colour.

6·8. Any manganous hydroxide separating out is centrifuged off. The solution at this stage should be practically colourless or of light straw colour and is ready for treatment with CNBr. Recovery of added nicotinic acid in solutions so treated is complete (Table 4). The advantage of this oxidation method is not limited to the production of an almost colourless extract. It gives also a solution free from reducing substances, such as ascorbic acid or sulphites, which when present in large amounts may prevent the proper development of colour. Other reducing substances so far tried (sodium thiosulphate, uric acid) had the same effect. The presence of such substances decreases the sensitivity of the method, and the advantage of the oxidation procedure is that it eliminates this interference. Hence the new modification seems to be a further improvement in comparison with other methods.

(III) *Comparison of different blanks*

Three different blanks were tried, (*a*) 'CNBr blank', i.e. a sample of urine treated in the same manner as the 'unknown' but without the amine; (*b*) 'amine blank', i.e. without CNBr; and (*c*) 'acid blank', see below.

Theoretically no blank can be considered perfect which omits any of the reactants. The 'CNBr blank' advocated by Bandier and described above, errs probably on the low side. The 'amine blank' of Harris & Raymond gives a higher correction, but the fact that CNBr is omitted might be criticized. It was found that when nicotinic acid was treated with CNBr in an acid solution, no reaction took place. This observation makes it possible to include all the reactants in the blank by simply adding acid to the test solution in the reversed order, namely, *before* treatment with CNBr. This blank is referred to in future as the 'acid blank'. It will be seen from Table 5 that the value of this blank is

Table 5. *Nicotinic acid estimated in urines with use of different blanks and charcoal decolorization*

	Nicotinic acid found (mg./day) with				
Sample	Urine blank*	CNBr blank	Amine blank	Amine blank plus CNBr blank	Acid blank
1	4·40	3·88	2·88	2·37	2·24
2	3·38	2·83	2·65	2·06	2·21
3	3·01	2·62	1·98	1·51	1·56
4	6·04	6·04	4·78	4·78	4·68
5	4·90	4·20	2·66	1·95	1·85
6	2·69	2·27	1·87	1·46	1·37
7	2·92	2·50	1·54	1·13	1·12
8	3·58	3·34	1·98	1·74	1·77
9	3·30	2·85	2·02	1·55	1·55
10	1·80	1·54	0·99	0·73	0·71

* Urine treated exactly in the usual manner, but without the addition of CNBr and amine reagent.

approximately equal to the 'CNBr blank' and the 'amine blank'. When the washing with isobutanol and the oxidation by $KMnO_4$ were used, the 'acid blank' showed even lower values, corresponding approximately to the sum of the original colour of the CNBr and amine. It is interesting to note that the values of normal human excretion of nicotinic acid obtained by means of this new blank tally well with those of Perlzweig *et al.* [1940] and of Snell & Wright [1941] determined by a different technique.

(IV) *Optimum conditions for the reaction of* CNBr *with nicotinic acid*

In their original method, Harris & Raymond used a temperature of 80° and a duration of 10 min. for the reaction. Kodicek [1940*b*] allowed 5 min. for the reaction to take place. The effect of both temperature and time was reinvestigated by varying these two factors.

(*a*) *Temperature.* A solution of pure nicotinic acid containing 2 μg./ml. was used. The CNBr was added and the reaction mixture placed for 5 min. on a water-bath maintained at constant temperature. The results are given in Table 6. It will be seen that the optimum temperature for maximal colour production is between 50 and 60°.

Table 6. *Effect of temperature on reaction of* CNBr *with nicotinic acid*

Temp. (° C)	Extinction
20	0·148
40	0·305
50	0·388
60	0·418
70	0·329
80	0·236

(*b*) *Time.* The same solution of nicotinic acid was used, and the temperature was kept at 56–58°. The reaction mixture was placed in the water-bath for varying lengths of time. As illustrated in Table 7, the highest extinction was obtained within 2–5 min. It was, therefore, decided to use 4 min. for the reaction in our procedure.

Table 7. *Effect of time on reaction of* CNBr *with nicotinic acid*

	Extinction	
Time (min.)	Exp. 1	Exp. 2
2·0	—	0·408
2·5	0·408	—
3·5	—	0·408
5·0	0·410	0·410
10·0	0·335	—
20·0	0·267	—

(V) *Optimum conditions for the development of colour*

(*a*) *Amount of amine.* It was felt that the amount of amine used previously by Harris & Raymond [1939] and Kodicek [1940 *a*, *b*] might not be the optimum amount for

Table 8. *Effect of variation in the amount of amine used*

10% ethanolic *p*-aminoacetophenone added (ml.)	Extinction for solution of nicotinic acid containing (μg.)	
	10	20
0·2	0·218	0·469
0·3	0·246	0·503
0·6	0·292	0·585
0·9	0·314	0·635
1·2	0·311	0·633
1·5	0·320	0·648

the reaction, as has already been pointed out [Kodicek, 1940*a*]. The fact that the urinary extract was different in composition from other extracts used by one of us (E.K.) made it necessary to reinvestigate this point. It is clear from Table 8 that a nearly optimum development of colour is reached with the addition of about 1·0 ml. of a 10% solution of *p*-aminoacetophenone in ethanol.

(*b*) *Effect of acid upon the final development of colour.* The intensity of colour produced by the reaction between the amine and the CNBr-treated nicotinic acid depends on the acidity of the solution. The optimal acidity corresponds to a pH of about 1·5, as illustrated by Table 9. It will be seen that the intensity of colour varies only slightly with changes of acidity below a pH value of 2. It was found that 0·6 ml. of a 10% HCl was sufficient to bring the pH of the urines so far examined to a value between 1 and 2 under the conditions of our procedure.

Table 9. *Effect of amount of acid*

10% HCl (ml.)	Extinction for 10 μg. nicotinic acid in *M*/45 phosphate buffer of pH 6·6	pH (quinhydrone electrode)
0·1	0·177	2·51
0·2	0·232	2·14
0·3	0·252	1·87
0·4	0·257	1·68
0·6	0·275	1·45
1·0	0·243	1·13

(*c*) *Stability of the colour produced.* It will be seen from Table 10 that the colour is fairly stable and there is very little fading after standing in the dark for periods up to 30 min. This agrees with the findings of Harris & Raymond [1939].

Table 10. *Fading of developed colour*

Time (min.)	Extinction for solution of nicotinic acid containing (μg.)		
	12·5	25	50
5	0·255	0·500	1·010
10	0·263	0·519	1·030
15	0·264	0·517	1·017
20	0·259	0·514	1·022
25	0·253	0·514	1·010
30	0·253	0·518	1·008
60	0·239	0·480	0·948

METHOD ADOPTED

Reagents

CNBr *solution.* Prepared fresh daily by titrating a saturated solution of ice-cold bromine water with 10% ice-cold NaCN solution until the colour of bromine disappears. (NaCN was found to be better than KCN.)

Alcoholic amine reagent. 10 g. of recrystallized *p*-aminoacetophenone were dissolved in 96% ethanol and made up to 100 ml. The solution should be prepared fresh every week as older solutions tend to deepen in colour. (The *p*-aminoacetophenone was recrystallized from a warm saturated solution in 96% ethanol by adding about 3 vol. of distilled water. After several hours at 0° the precipitate was filtered off. The process of crystallization may be repeated a second time if necessary. The purified product was dried *in vacuo* over $CaCl_2$. It should be of a light creamy colour.)

HCl *solution* 10% w/w.

Concentrated HCl *solution* 36% w/w.

NaOH *solution* 40% w/v.

$KMnO_4$ *solution* 4% w/v.

Isobutanol. Redistilled and saturated with water.

Standard solution of nicotinic acid. 20 μg./ml. in 25% ethanol. Prepared weekly from a stronger solution containing 1 mg./ml. in 25% ethanol.

Procedure

(1) *Preliminary digestion.* If the total amount of urine excreted each day is less than 1500 ml., dilute the urine to this volume. Take 20 ml. of urine, add 2 ml. of 40% NaOH and heat on a boiling water-bath for 45 min. Cool, adjust pH to 2–3 with conc. HCl, with thymol blue as external indicator. Make up the volume to 25 ml. with distilled water. Pour the treated urine into a 50 ml. separating funnel; add isobutanol corresponding to one-half the final volume of digested urine and shake for 2 min. Allow to separate, then run out the aqueous layer. Take 20 ml. of this water layer and evaporate the isobutanol present in the solution on a boiling water-bath for about 15 min. in an open beaker. Some isobutanol is still left in the solution, but as it does not interfere with the final estimation, its complete removal is not necessary. Add to the hot solution 15 ml. of distilled water, and cool it down to about 40–50°. Acidify further with 6 drops of concentrated HCl and oxidize by adding a 4% solution of $KMnO_4$ drop by drop with continuous stirring, until the urine is completely decolorized and the pink colour of the permanganate stays for more than 30 sec. Allow the solution to cool. Stand for about 15 min. to ensure that any excess of $KMnO_4$ is completely reduced, then neutralize cautiously with 40% NaOH to pH 6·5–6·8 with bromothymol blue as external indicator. It is essential that the pH 6·8 should not be overstepped, as an alkaline reaction causes a brownish coloration. Make up to 50 ml. with distilled water and centrifuge off any precipitate that is formed.

(2) *Reaction with* CNBr. Four graduated 15 ml. flasks are labelled A, B, C and D. To flasks C and D add 0·5 ml. of diluted standard nicotinic acid solution. To flask A add 0·6 ml. of 10% HCl; this flask is to be used as a blank. Measure 10 ml. of the urine solution into each of the four flasks. Mix well, especially flask A, then add to all four flasks 2 ml. of CNBr solution. After the mixing, place the flasks for 4 min. in a water-bath kept at 56–58°. Cool the flasks for 5 min. in tap water (temperature 18–20°) in a darkened place.

(3) *Development of colour.* To each flask add 1 ml. of the alcoholic amine reagent and shake. Then to flask B, C and D add 0·6 ml. of 10% HCl. Fill the flasks up to the 15 ml. mark with distilled water. Mix the contents and allow to stand for 5 min. in a darkened place. Immediately afterwards take measurements with the Pulfrich photometer, using 3 cm. cells and filter S 47 [Kodicek, 1940*a*, *b*]. Flask A is used as the blank, and the flasks B, C and D are examined for their contents of nicotinic acid.

(4) *Calculation of result.* The relation between intensity of colour (extinction) and nicotinic acid present is linear [Harris & Raymond, 1939]. Hence, nicotinic acid content, μg./ml. of urine, equals

$$\frac{B-A}{(C-A)-(B-A)} \times S \times \frac{F}{10} \times 1{\cdot}1 \times \frac{25}{20} \times \frac{1}{V_0}$$

$$= \frac{B-A}{C-B} \times S \times \frac{F}{V_0} \times 1{\cdot}375.$$

When, according to the procedure, $S = 10$ μg., $F = 50$ ml. and $V_0 = 20$ ml., the formula is simplified to $\frac{B-A}{C-B} \times 3{\cdot}44$ μg./ml. of urine. A = extinction of solution in flask A (blank), B = extinction of solution in flask B (urine), C = mean of extinctions of solution in flasks C and D (urine + added nicotinic acid), S = μg. of nicotinic acid added to flasks C and D, F = final volume of urine after washing with isobutanol, oxidation and neutralization, V_0 = original volume of urine taken for analysis, 1·1 = factor allowing for the increase in volume of washed urine due to isobutanol.

Reproducibility of method

To test the reproducibility of the method separate determinations were done on one specimen of urine. The results are shown in Table 11. The standard error of a single determination is about ±2·4%. This degree of precision is highly satisfactory.

Table 11. *Data testing the reproducibility of method*

Exp. no.	Nicotinic acid in same specimen of urine (μg./ml.)
1	0·81
2	0·86
3	0·85
4	0·82
5	0·81
Average	0·83

Standard deviation of single determination ±0·02 (2·4%).

RESULTS

Daily excretion of nicotinic acid by normal individuals determined by the new procedure

Ten normal subjects were examined for their daily excretion of nicotinic acid under standard conditions (see below). The final modification described above was used. The resting levels of adults varied from 0·85 to 2·33 mg. of nicotinic acid/day, with an average of 1·15 mg. (Table 12). These figures agree with the values found by the earlier procedure of charcoal decolorization.

Table 12. *Daily excretion of nicotinic acid by normal individuals as determined by the final modification*

Subject	Age	Sex	Daily volume of urine (ml.)	Nicotinic acid excreted (mg./day)
J.E.P.	17	F	880	0·85
E.M.C.	41	F	1450	1·03
J.Y.	30	M	1360	1·14
L.W.M.	33	M	1400	1·16
A.G.D.	17	M	1040	1·43
E.K.	33	M	1100	1·53
S.G.I.	30	M	2160	1·77
T.M.	41	M	2350	1·92
D.W.	19	M	1630	2·33
J.K.	4	F	650	0·46

*Excretion after experimental dosing of healthy subjects with nicotinic acid**

Four normal subjects living on their usual diets were examined for their urinary excretion of nicotinic acid. The daily output of urine was collected in two portions, a morning 3 hr. sample and the remaining 21 hr. portion; each portion was analyzed separately. Test doses were taken with or after breakfast. Table 13 gives the results of the experiment.

The average daily excretions of nicotinic acid by the four individuals were 1·09, 1·42, 1·47 and 2·10 mg., and the corresponding morning 3-hourly outputs averaged 0·23, 0·09, 0·13 and 0·16 mg. respectively. Smoking did not appreciably change the values. After daily ingestion of 100 mg. of nicotinic acid for several days, the average daily excretions of the last three individuals increased to 7·77, 3·98 and 9·51 mg., and the corresponding 3-hourly excretions to 5·34, 2·02 and 6·38 mg. respectively.

It will be seen from Table 13 that while the excretions of nicotinic acid by the different subjects on their usual diets changed very little from day to day and varied little from one to another, the outputs after ingestion of test doses fluctuated considerably,

* Decolorization of urine in these experiments had been performed by charcoal adsorption as described in § II(*a*). Urine collected after dosing had, however, such a high concentration of nicotinic acid that the charcoal adsorption could be replaced by a simple dilution of the urine.

Table 13. *Response of normal individuals to test doses of nicotinic acid*

Subject	Day	Test dose (mg.)		Nicotinic acid excreted (mg.) in 3 hr.	Nicotinic acid excreted (mg.) in 24 hr.	Notes
Y.L.W.	1	0		—	2·24	No smoking
Body weight	2	0		—	2·21	,,
65 kg.	3	0		—	1·56	,,
	4	0		—	1·26	Smoking
	5	0		0·05	1·21	,,
	6	0		0·12	0·71	,,
	7	0		0·10	1·40	,,
	8	0		0·08	0·77	,,
			Average	0·09	1·42	
	9	100		2·91	4·68	
	10	100		5·16	8·38	
	11	100		2·34	3·74	
	12	100		2·98	4·92	
	13	100		16·30	21·20	
	14	100		2·32	3·70	
			Average	5·34	7·77	
L.J.H.	1	0		—	0·63	No smoking
Body weight	2	0		—	2·21	,,
72 kg.	3	0		—	2·16	,,
	4	0		—	2·56	Smoking
	5	0		—	1·90	,,
	6	0		0·14	2·06	,,
	7	0		0·12	1·25	,,
	8	0		0·17	1·09	,,
	9	0		0·20	1·91	,,
			Average	0·16	2·10	
	10	100		1·33	3·07	
	11	100		5·16	7·82	
	12	100		4·48	7·60	
	13	100		17·50	23·98	Dose taken on empty stomach
	14	100		2·00	4·28	
	15	100		7·82	10·30	
			Average	6·38	9·51	
	16	0		0·53	2·30	
	17	0		0·17	1·27	
	18	0		0·59	4·39	
E.K.	1	0		—	2·50	No smoking
Body weight	2	0		—	1·85	,,
87 kg.	3	0		—	1·37	,,
	4	0		—	1·12	Smoking
	5	0		—	1·77	,,
	6	0		0·10	0·82	,,
	7	0		0·15	1·00	,,
	8	0		0·14	1·48	,,
	9	0		0·14	1·28	,,
			Average	0·13	1·47	
	10	100		1·13	2·58	
	11	100		1·75	2·98	
	12	100		1·49	2·47	
	13	100		0·61	2·32	
	14	100		4·60	9·20	
	15	100		2·24	3·81	
	16	100		2·35	4·47	
			Average	2·02	3·98	
	17	0		1·22	3·08	
	18	0		0·11	2·53	
G.G.	1	0		—	1·55	
Body weight	2	0		—	0·71	
83 kg.	3	0		—	0·92	
	4	0		—	0·91	
	5	0		—	0·77	
	6	0		0·16	0·71	
	7	0		0·10	0·86	
	8	0		0·42	1·55	
	9	0		0·24	1·85	
			Average	0·23	1·09	

making it difficult to draw any definite conclusion from the experiment. It will be noted also that the different individuals varied a great deal in their response to dosing. Immediately after the cessation of dosing, the excretion of nicotinic acid returned to the previous level.

In view of the big fluctuations in excretion after the intake of test doses, it was thought advisable to standardize the conditions under which the doses were administered. Three subjects were given a special breakfast which contained little nicotinic acid or trigonelline, and consisted of two cups of tea, two slices of white bread, jam, butter and about 50 ml. of milk, and the dose given with the breakfast after one cup of tea and one slice of bread. The urines were collected and analyzed as before, and the results are given in Table 14. It will be seen that assessing the body reserves of the vitamin, they must be administered under a standardized condition, otherwise little information can be gained from the results.

Time curve of excretion of ingested nicotinic acid under uniform conditions

In one normal subject the hourly excretion of nicotinic acid after ingestion of 100 mg. of nicotinic acid was studied under standardized conditions. The excretion was followed at hourly intervals for 5 hr. (Table 15). Our findings, similar to those of Bandier [1939*a*], were that 91 % of the administered nicotinic acid was excreted in the first 5 hr. after the dose, and that the maximum excretion occurred in the first hour and amounted to about 63 %. This rapid elimination suggests that the organism is not

Table 14. *Response of normal individuals under standardized conditions of dosing*

Subject	Day	Test dose of nicotinic acid (mg.)		Nicotinic acid excreted (mg.) in 3 hr.	Nicotinic acid excreted (mg.) in 24 hr.
Y.L.W.	1	0		0·12	1·20
Body weight	2	0		0·16	1·21
65 kg.			Average	0·14	1·21
	3	100		9·70	12·28
	4	100		11·20	13·80
	5	100		8·40	—
			Average	9·97	13·04
	6	0		0·21	6·61
	7	0		0·18	1·68
	8	0		0·15	0·90
E.K.	1	0		0·41	1·64
Body weight	2	0		0·11	1·40
87 kg.			Average	0·26	1·52
	3	100		0·85	2·36
	4	100		1·27	3·31
	5	100		1·03	11·03
			Average	1·05	5·57
	6	0		0·25	1·90
	7	0		0·25	1·99
	8	0		0·22	1·05
G.G.	1	0		0·16	0·71
Body weight	2	0		0·10	0·86
83 kg.	3	0		0·42	1·55
	4	0		0·24	1·85
			Average	0·23	1·26
	5	100		10·89	13·69
	6	100		9·85	11·17
	7	100		11·20	13·93
			Average	10·65	12·93
	8	0		0·20	1·40
	9	0		0·10	1·23

the 3-hourly excretions after dosing were fairly constant from day to day, and also the 24 hr. outputs did not fluctuate as before when the conditions of dosing were not standardized. It is obvious that if test doses of nicotinic acid are to be used for able to utilize efficiently all the ingested nicotinic acid and therefore excretes the greater part of it promptly. This phenomenon does not occur when our subjects are dosed with nicotinic amide. Both the acid and the amide, however, produce a large

Table 15. *Rate of excretion of ingested nicotinic acid*

Time	Nicotinic acid excreted (mg./hr.)
3 hr. period before dosing	0·23
Total 24 hr. period before dosing	1·26
100 mg. nicotinic acid given by mouth	
0 hr.	—
1	7·77
2	2·46
3	0·66
4	0·37
5	0·25
5 hr. total excretion	11·51
5–24 hr.	2·18
24 hr. total excretion	13·69

increase in the excretion of trigonelline and related N-methyl pyridinium compounds which can be regarded as normal metabolites. The fate of ingested nicotinic acid or amide in the body has been briefly discussed in our previous report [Kodicek & Wang, 1941]. The detailed results will be published in a subsequent paper. On the basis of these findings, we have recommended dosing with nicotinic amide, followed by the estimation of the urinary excretion both of trigonelline and nicotinic acid, as a method of assessing the level of nutrition with regard to the anti-pellagra vitamin.

SUMMARY

1. The cyanogen bromide-*p*-aminoacetophenone method of Harris & Raymond for the estimation of nicotinic acid in urine has been further studied, and several additional improvements have been introduced, viz. (*a*) decolorization of the digested urine by washing with isobutanol and oxidation with $KMnO_4$; (*b*) the use of a lower temperature, a shorter time for the reaction of CNBr with nicotinic acid, and the addition of larger amounts of *p*-aminoacetophenone to the reaction mixture; and (*c*) the introduction of a modified blank.

2. The normal excretion of nicotinic acid by ten healthy subjects and the response to test doses of nicotinic acid by four of them has been studied.

3. The maximum excretion of ingested nicotinic acid occurs in the first three hours, the first hour showing the highest output.

4. It is emphasized that when test doses of nicotinic acid are used for assessing the body reserves of the vitamin, they should be administered under standardized conditions.

5. For obtaining reliable information of the nutritional status in regard to the anti-pellagra vitamin, dosing with nicotinic amide is to be preferred to dosing with nicotinic acid, and estimation should then be made of the excretion of trigonelline, as well as that of other substances related to nicotinic acid.

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The Etherification of Hydroxyamino-acid Residues in Silk Fibroin by Dimethyl Sulphate

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In recent years it has been recognized that in addition to tyrosine the hydroxyamino-acids serine and threonine are major protein constituents. The mode of linkage of the —OH groups of these latter in the protein is of interest, since such groups might play a part in non-peptide linkage of amino-acid residues

[Chibnall, 1942; Linderstrøm-Lang & Jacobsen, 1941], or, if free, influence significantly the chemical and physical properties of the protein. Colour reactions and spectroscopy have thrown considerable light on the mode of incorporation in proteins of the phenolic —OH groups of tyrosine, but these methods are not of value in studying aliphatic —OH groups. The acylation of proteins has been studied by numerous workers, but, while the number and lability of acyl groups introduced provides circumstantial evidence for the occurrence of free —OH groups, the lability of *O*-acyl groups to the usual hydrolytic agents has precluded confirmation of this by the isolation of *O*-acylated compounds from hydrolysates of acylated proteins.

Methylation has been extensively and successfully employed for investigating the mode of linkage of aliphatic —OH groups in carbohydrates. Many workers, for a variety of purposes, have applied methylating agents to proteins [Blackburn, Carter & Phillips, 1941], but few [e.g. Skraup & Krause, 1909; Haurowitz, 1938] have employed conditions suitable for methylating aliphatic —OH groups, and of these none seems to have identified *O*-methylated compounds after hydrolyzing the protein. The strongly alkaline aqueous conditions, which appear necessary for the methylation of aliphatic —OH groups in proteins, induce degradative changes in the protein, making interpretation more complicated. Nevertheless, it seemed worth attempting the application of this technique in the protein field.

We have studied the action of dimethyl sulphate on silk fibroin in the presence of excess cold aqueous NaOH. This protein was chosen because of its resistance to solution by cold alkaline reagents, which facilitates repeated treatment, its negligible S content and its high content of hydroxyamino-acids.

Repeated treatment of silk fibroin in this way introduces *O*-methyl groups to an extent which cannot be increased by continued treatment. At this stage nearly all the tyrosine residues and about one-half the serine residues have been converted to their *O*-methyl derivatives. Our evidence for this is: (1) Disappearance of those colour reactions (Millon, xanthroproteic) due to free —OH groups of tyrosine residues from methylated fibroin and its hydrolysates. (2) Diminution of the hydroxyamino-acid content (NH_3 liberated by periodate) of hydrolysates of methylated fibroin to about one-half of the corresponding value for normal fibroin. (*O*-methyl-serine does not give NH_3 with periodate.) (3) The —OMe content of methylated fibroin and its hydrolysates corresponds stoichiometrically to the tyrosine + half the hydroxyamino-acid content of fibroin. (4) The occurrence of *O*-methyl-tyrosine and *O*-methyl-serine in hydrolysates of methylated fibroin. These have been demonstrated in the expected quantities, after conversion to their *N*-acetyl derivatives. For the latter compound we have employed partition chromatography on silica gel [cf. Gordon, Martin & Synge, 1943*a*].

Preliminary acetylation of the fibroin by boiling acetic anhydride [cf. Carr, 1938*b*] was found not to increase the amount or rapidity of methylation. Preliminary acetylation has been found useful in the methylation of polysaccharides [cf. Hirst & Young, 1939].

It was found that fully methylated fibroin on subsequent acetylation took up correspondingly fewer *O*-acetyl groups (determined by saponification with cold alkali) than normal fibroin. However, some acetyl groups saponifiable under these conditions were obtained on acetylation of fully methylated fibroin.

From the above it is apparent that the reaction of dimethyl sulphate with silk fibroin in the presence of alkali is entirely different from that occurring in its absence, which, according to Blackburn *et al.* [1941], leads to the introduction mainly of ester-type *O*-methyl which can be entirely liberated by the action of dilute alkali. Rutherford, Patterson & Harris [1940], by exhaustive treatment of fibroin with diazomethane in ether, obtained a product in which (as judged by non-isolative procedures) the tyrosine-phenolic groups were completely etherified, but there was no evidence for methylation of aliphatic —OH groups, most of the extra *O*-methyl being of a type similar to that studied by Blackburn *et al.* [1941].

Since the amino-N content of a hydrolysate of our fully methylated fibroin was little different from that of a hydrolysate of untreated fibroin, it seems unlikely that the reagents employed gave rise to significant *N*-methylation of amino-acid residues in peptide linkage.

Since the positions in the fibroin at which *O*-methyl groups are introduced by methylation appear also to be accessible to acetylation by a procedure [cf. Carr, 1938*b*] that does not seriously degrade the fibroin, it is reasonable to conclude that these (i.e. the —OH groups of nearly all the tyrosine and about one-half of the serine residues) are free in intact fibroin. For the tyrosine, this conclusion is consistent with the phenolic colour reactions of fibroin, and with the data of Rutherford *et al.* [1940] on methylation, of Meyer, Fuld & Klemm [1940] on coupling with diazo compounds, and of Kise & Carr [1936] and Carr [1938 *a*, *b*] on acetylation. Serine residues having free —OH groups might be expected to take part in the alkaline degradation and coupling reactions demonstrated in whole silk by Nicolet & Shinn [1941 *a*, *b*].

The mode of linkage of the serine residues which do not undergo *O*-methylation remains obscure. The present work has not established whether or

not their —OH groups are accessible to acetylation. It is possible that they are not methylated owing to incorporation in 'crystallites' in the fibre, while serine residues in the 'encrusting substance' are reactive [cf. Meyer *et al.*, 1940; Synge, 1943; Huggins, 1943]. Or they might be involved in oxazoline rings of the type postulated by Linderstrøm-Lang & Jacobsen [1941] [cf. Blackburn, Middlebrook & Phillips, 1942]. The present work only demonstrates that the serine residues of silk fibroin fall into two distinct categories with respect to their reactivity with dimethyl sulphate and alkali in the cold. Investigation of the behaviour under these conditions of other proteins and model substances, though technically more difficult owing to their solubilities, may help to elucidate this question.

EXPERIMENTAL

Analytical methods

Estimation of tyrosine. The colorimetric method of Arnow [Block, 1938] was employed.

Estimation of total hydroxyamino-acids. The method of Van Slyke, Hiller & MacFadyen [1941] was modified, by the use throughout of one-tenth of the amounts of reagents which they employed, and by omission of the capryl alcohol. The reagents were added to the outer compartment of the Conway micro-diffusion apparatus. The NH_3 evolved after $1\frac{1}{4}$ hr. gentle rotatory shaking was determined by absorption in 1·0 ml. of 1% boric acid mixture, according to the method of Conway & O'Malley [1942]. Under these conditions we obtained 94–95% recovery of NH_3 (80–120μg.) from ammonium sulphate in 2 ml. half-saturated K_2CO_3 solution. All results have been corrected for this incomplete absorption. Serine under these conditions yielded the theoretical quantity of NH_3.

Estimation of —OMe. On methylated fibroin and its hydrolysates this was determined by Dr S. Blackburn with the volatile iodide procedure of Baernstein [cf. Blackburn *et al.*, 1941]. Other determinations were made with the standard Pregl procedure by Dr G. Weiler, Oxford, who also carried out carbon and hydrogen determinations.

Estimation of total N. Semimicro-Kjeldahl determinations were employed, using the incineration mixture of Campbell & Hanna [1937].

Estimation of amino-N. The manometric Van Slyke procedure (4 min. reaction time) was employed. Where the sample contained free mineral acid, this was neutralized before analysis with excess of sodium acetate.

Melting points are given uncorrected. Optical rotations were observed in a 2 dm. tube.

Material

Material from the same batch of unweighted silk fabric as was studied by Blackburn *et al.* [1941] was employed throughout this work. The fabric was thoroughly rinsed with cold distilled water before use. Weights were determined after samples had been dried to constant weight over H_2SO_4 at room temp. *in vacuo*.

Methylation of fibroin

A typical experiment is described: 3·284 g. of fabric were cut into small pieces and placed in a bottle with 40 ml. of *N* NaOH solution. 0·75 ml. of dimethyl sulphate was added. At 10 min. intervals 20 ml. *N* NaOH solution and 0·75 ml. dimethyl sulphate were added, until altogether 7·5 ml. dimethyl sulphate had been added. The preparation was shaken gently at room temperature the whole time.

At the end, most of the liquor was poured from the fabric, and treatment was repeated. After each treatment a test portion of the fabric was placed in sodium nitrite-acetic acid solution, and the development of yellow colour was observed. This steadily decreased until after four treatments it was practically absent.

When the preparation had to be left between treatments, the fabric was suspended in dilute acetic acid.

Samples removed for determination of —OMe were washed in six changes of distilled water before analysis. The course of methylation is shown in Table 1.

Table 1. *Course of methylation of silk fibroin*

No. of treatments.	*O*-methyl content (CH_3 as percentage of dry wt. of product)
4	1·95
6	2·09
9	2·28
12	2·15

It appears that *O*-methylation is substantially complete after six treatments. The weight of final product (12 treatments), together with that of samples removed during methylation, was 2·96 g. The original material had N, 18·3%, and the final product N, 17·6%. Recovery, based on the N content, was therefore about 85%.

After treatment the fabric was readily creased, had much lower tensile strength, and lacked lustre.

Methylation following acetylation of fibroin

Dry fibroin was acetylated by refluxing in excess of acetic anhydride for $\frac{1}{2}$ hr. The reagent was then washed off with water, and the material subjected to a single methylation treatment as above. The material was then rinsed, dried, and again subjected to acetylation. After four such cycles the product had *O*-methyl (CH_3 as percentage of dry weight) 1·64%, suggesting that the method has no advantages over direct methylation, especially since greater losses resulted.

Acetylation of normal and methylated fibroin

The fabrics were acetylated by refluxing for $\frac{1}{2}$ hr. in acetic anhydride, rinsed off with several changes of distilled water and dried.

The difference between the alkali neutralized by acetylated and unacetylated material on suspending in cold dilute alkali served as a measure of the *O*-acetyl groups introduced.

Approximately 60 mg. of fabric were placed in a stoppered flask in 10 ml. *N*/50 $Ba(OH)_2$, and kept at room temperature with occasional gentle agitation. Control experiments showed that under these conditions no further alkali was neutralized after 6 hr. After 24 hr. a measured portion of fluid was removed from the flask and titrated with dilute HCl, with the exclusion of CO_2 and with phenolphthalein as indicator. Calculated for the whole volume, the difference from the direct titration of the alkali gave the total base bound by the saponified fibroin together with that neutralized by the acetic acid liberated in the saponification. The results of these experiments are shown in Table 2.

Table 2. *Analyses of silk fibroin after various treatments*

Material	Equivalents expressed in terms of N (1 atom) as a percentage of total N of material analyzed: Untreated material	Same acetylated	Methylated (12 treatments)	Same acetylated
Alkali neutralized	5·1	18·4	1·9	6·0
O-methyl content	0·0	0·0	11·4	12·4

Thus for unmethylated fibroin the *O*-acetyl groups introduced are 18·4 − 5·1 = 13·3% (equiv. in terms of N as a percentage of total N of fibroin), while for methylated fibroin the corresponding figure is 6·0 − 1·9 = 4·1%. The difference of *O*-acetyl content (13·3 − 4·1 = 9·2%) is in reasonable agreement with the *O*-methyl content of the methylated fibroin, shown in Table 2, where it is expressed in the same units. The decreased binding of alkali by methylated as compared with untreated fibroin is presumably due to methylation of the base-binding phenolic groups of tyrosine residues [Gleysteen & Harris, 1940].

The yields of insoluble material after methylation, acetylation and saponification, expressed on a N basis, are shown in Table 3.

Table 3. *Yields and analyses of fibroin after various treatments*

Treatment	Yield (total N of product as percentage of total N of untreated fibroin)	*O*-methyl content (—OMe (1 equiv.) expressed in terms of N (1 atom) as percentage of total N of product)
Untreated	100	—
Untreated after saponification	96·7	—
Methylated	86	11·4
Same material after saponification	83	12·6
Acetylated	97	—
Same material after saponification	85·5	—
Methylated and then acetylated	73·6	12·4
Same material after saponification	65·5	11·2

The relative constancy of the *O*-methyl content of the material (Table 3) despite the considerable losses encountered in these treatments suggests that these losses did not involve serious qualitative fractionation of the fibroin, and justifies the assumption that the material lost by solution during processing was not chemically very different from that retained.

Hydrolysis of methylated fibroin

Refluxing fully methylated fibroin for 24 hr. in 6 *N* HCl resulted in considerable de-methylation. Tyrosine colour reactions appeared in the hydrolysate. The —OMe content of the hydrolysate (determined after evaporation to dryness) was 2·3% (—OMe expressed in terms of N (1 atom) as percentage of total N of material), compared with a figure of 11·4% for the material before hydrolysis. The NH_2-N liberated by periodate was 10·0% of the total N of the hydrolysate, the corresponding figure for a hydrolysate of untreated fibroin being 10·9%.

Hydrolysis of methylated fibroin for 24 hr. in boiling 6 *N* NaOH reduced the —OMe content (same units—determined after neutralization of hydrolysate with HCl and evaporation to dryness) to 5·1%. Destruction of *O*-methyl-tyrosine would not be expected under these conditions [Rutherford *et al.*, 1940], but when 35 mg. of *O*-methyl-*dl*-serine were refluxed for 24 hr. in excess 6 *N* NaOH with 500 mg. of untreated silk fibroin, only 26%, as measured by an —OMe determination, remained.

Bell [1937] noted removal of ether-methyl during treatment of methylated glycogen with hot hydrochloric acid, and found that *p*-toluenesulphonic acid effected satisfactory hydrolysis without de-methylation. For the present work we have found sulphuric acid to be satisfactory. 70% of the —OMe of 37 mg. *O*-methyl-*dl*-serine was recovered after refluxing with 200 mg. of untreated silk fibroin in excess 6 *N* H_2SO_4 for 24 hr. ($SO_4^=$ was removed from the hydrolysate exactly by precipitation with $BaCl_2$. The filtrate and washings were then evaporated to dryness *in vacuo* at room temperature before analysis.)

Analysis of hydrolysates of methylated and untreated fibroin

For comparative purposes, both methylated and untreated fibroins were hydrolyzed by refluxing for 24 hr. in 30 parts by weight of 6 *N* H_2SO_4. $SO_4^=$ was removed by addition of the calculated quantity of $BaCl_2$ in aqueous solution, and the filtrate and washings from $BaSO_4$ were evaporated to dryness *in vacuo* below 40° and made up to known volume in water before analysis. The results are given in Table 4.

Demonstration of O-methyl-tyrosine in hydrolysate of methylated fibroin

O-methyl-tyrosine was obtained as its *N*-acetyl derivative by direct extraction and crystallization after acetylation of the hydrolysate [cf. Synge, 1939].

Methylated fibroin containing 234 mg. N (OMe as N (1 atom) in % of total N, 11·4%) was refluxed for 24 hr. in 25 ml. 6 *N* H_2SO_4. The resulting hydrolysate was neutralized with NaOH to thymol blue (yellow), concentrated *in vacuo* until crystallization set in, made alkaline by addition of further NaOH and acetylated at 0° by addition of 5 ml. of acetic anhydride and 50 ml. 2 *N* NaOH added in portions with shaking. After keeping alkaline 2–3 hr. the solution was acidified to thymol blue (pink) with 10 *N* H_2SO_4 and extracted five times with 125 ml. portions of B.P. chloroform. The combined chloroform extracts were evaporated to dryness. Crystallization of the residue from water yielded gummy crystals, which were freed from gum by two recrystallizations from chloroform. The final product (61 mg.) had m.p. 147°, not depressed on admixture of authentic *N*-acetyl-*O*-methyl-*l*-tyrosine, $[\alpha]_D^{20°} + 51·5°$ (ethanol, $c = 0·9$). Synge [1939] records for *N*-acetyl-*O*-methyl-*l*-tyrosine m.p. 151°, $[\alpha]_D^{20°} + 54·3°$, $+54·4°$ (ethanol, $c = 1·3$). (Found: C, 61·0; H, 6·53; N, 4·8; OMe, 12·4%; acid equiv. wt. 250. Calc. for $C_{12}H_{15}O_4N$: C, 60·8; H, 6·33; N, 5·9; OMe, 13·1%; acid equiv. wt. 237.)

Table 4. *Analytical data on hydrolysates of methylated and untreated fibroins*

	Percentage of total N of material analyzed			
		Untreated fibroin		
	Methylated fibroin	Present work	Other authors	Reference
	Before hydrolysis			
—OMe (1 equiv. = 1 atom N)	10·3	—	—	
	After hydrolysis			
(1) —OMe (1 equiv. = 1 atom N)	9·15	—	—	
(2) Amino-N	90	92	—	
(3) Tyrosine N	0·35	4·5	4·6*	Meyer, Fuld & Klemm [1940]
(4) NH_3-N + NH_3-N liberated by periodate	7·0	11·9	—	
(5) NH_2-N	1·45	1·0	—	
(6) Hydroxyamino-acid N (by difference of (4) and (5))	5·55	10·9	10·8*	Nicolet & Saidel [1941]

* N content of the fibroin analyzed by these workers assumed to be 18·3%.

Demonstration of O-methyl-serine in hydrolysate of methylated fibroin

O-methyl-serine was separated as its *N*-acetyl derivative by partition chromatography on silica gel [Gordon *et al.*, 1943*a*]. Control experiments with authentic *N*-acetyl-*O*-methyl-*dl*-serine [Synge, 1939] showed that on partition chromatograms employing B.P. chloroform, ethyl acetate or 30% propanol-cyclohexane as developing solvent, it is not separable from acetylalanine. With 17% butanol-chloroform, *N*-acetyl-*O*-methyl-serine travels as a band sufficiently faster than acetylalanine to permit the recognition of its presence, though not fast enough to permit satisfactory separations. However, with 1% butanol-chloroform, it moves as a separate band having *R* [Martin & Synge, 1941] approximately 1·5 times that of acetylalanine.

On the basis of this information the following experiments were carried out: Approximately 10 mg. samples of fully methylated (as used in previous section) and untreated fibroin were hydrolyzed with sulphuric acid, and the hydrolysates evaporated to dryness *in vacuo* below 40° after removal of SO_4^{--} as described above. The hydrolysates were then analyzed according to the procedure given by Gordon *et al.* [1943*a*]. Pelargonin chloride was employed as indicator. During the development with 17% butanol-chloroform it was noted that with the material from methylated, as compared with that from untreated fibroin, the bands corresponding to *N*-acetyl-tyrosine and *N*-acetyl-serine [Gordon, Martin & Synge, 1943*b*] were greatly reduced in intensity, while the band corresponding to acetylalanine was broader and exhibited a 'faint front', as with the synthetic mixtures of acetylalanine and *N*-acetyl-*O*-methyl-serine studied above. The bands corresponding to acetylglycine did not differ in appearance.

The values found for 'alanine' (N as percentage of total N of fibroin) were 24·5% for untreated and 30·8% for methylated fibroin. The difference (6·3%) is consistent with the *O*-methyl-serine value suggested by the data given above. Bergmann & Niemann [1937] recorded for alanine in silk fibroin a corresponding figure of 21·8%. They assumed their fibroin to contain 19·0% N.

After titration the 'alanine' fractions were acidified and examined on 1% butanol-chloroform chromatograms. That from methylated fibroin showed two bands, similar to those given by the synthetic mixtures described above, while that from untreated fibroin gave a single band (acetylalanine).

An attempt was then made to isolate *O*-methyl-serine as its acetyl derivative. Since the only authentic material available for comparison [Synge, 1939] was racemic, the acetylation was conducted under racemizing conditions [du Vigneaud & Meyer, 1932]. Complete racemization was not, however, achieved, and the crystallization of the final product may have been interfered with by partial racemization thus induced.

275 mg. of the same methylated fibroin were hydrolyzed with sulphuric acid, freed from SO_4^{--} with $BaCl_2$ and evaporated to dryness as above. The product was treated with 5 ml. 2·4*N* NaOH and 2 ml. acetic anhydride, the mixture being kept at 40° for 15 hr. The mixture was then cooled, made alkaline to thymolphthalein by addition of 12 ml. 3*N* NaOH, and kept at room temperature for 2 hr. to saponify *O*-acetyl groups. The mixture was then acidified with 10*N* H_2SO_4 and extracted with five 100 ml. portions of 17% butanol-chloroform. The material in the combined extracts was subjected to chromatographic analysis with 17% butanol-chloroform as developing solvent. The resulting 'alanine' band was subjected to further analysis, by development with 1% butanol-chloroform. Of the two bands so obtained, the faster yielded 20 mg. of material. Treated with ethyl acetate-light petroleum this yielded 11 mg. of partly crystalline gummy material having m.p. 128–130°, $[\alpha]_D^{22°} - 12°$ (ethanol, $c = 0{\cdot}4$). The material depressed the m.p. of acetyl-*l*-alanine (m.p. 126°) and of acetyl-*dl*-alanine (m.p. 133°) on admixture by more than 30°. It proved difficult to recrystallize. OMe was determined on the material recovered from the polarimetric determination after removal of ethanol by evaporation *in vacuo* over H_2SO_4. (Found: OMe, 7·3%. Calc. for $C_6H_{11}O_4N$: OMe, 19·3%.)

SUMMARY

1. Repeated treatment of silk fibroin with dimethyl sulphate in *N* NaOH at room temperature results in *O*-methylation of nearly all its tyrosine residues and about one-half of its serine residues.

2. The groups accessible to etherification in this

way are also accessible to acetylation when the fibroin is treated with boiling acetic anhydride.

3. The existence is thus established of two categories of serine residue in silk fibroin, differing in their accessibility to etherification by dimethyl sulphate.

We are grateful to Dr Herbert E. Carter for a gift of *O*-methyl-*dl*-serine.

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The Relative Efficacy of Calcium Carbonate and Calcium Phosphate in Preventing Rickets in Rats

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Present knowledge of the metabolism of calcium and phosphorus suggests that calcium phosphate is likely to be more effective as a calcifying agent than the carbonate. Since the question of dietary supplies of calcium became topical with the proposal to add carbonate to bread, some experiments were begun to compare the power of the carbonate and the phosphate to prevent rickets in rats. The applicability of the findings to human rickets will be discussed after the presentation of the results.

EXPERIMENTAL

General plan. The general plan of the experiments was to place young rats on diets comprising mostly flour to which were added varying amounts of $CaCO_3$ or $CaHPO_4$; after 5 weeks on these diets the ash content of both femurs of each animal was determined. The rats at the beginning of the experiment were about 6 weeks old and weighed about 50 g.; they were kept in separate cages throughout the experiment and weighed weekly. Each group receiving a given level of Ca contained five animals. $CaCO_3$ was given as the preparation used to fortify bread, i.e. *creta preparata* B.P. The highest amount of each salt added to the diet was equivalent to 3% of Ca, i.e. 7·5% $CaCO_3$ or 10·1% $CaHPO_4$.

Diets. In most of the experiments the basal diet consisted of flour to which was added 1% NaCl. The flour was either white (70% extraction) or National Wheatmeal (85% extraction). When white flour was used the rats were given supplements of aneurin and riboflavin equivalent to 10μg. daily of the former and 4μg. of the latter. No supplement was given with the wheatmeal flour. For comparative purposes one experiment was carried out with a maize diet similar to that commonly used for producing rickets in rats. This basal diet contained maize meal 76 parts, gluten 20 parts.

To the basal flour or maize diets were added 1% NaCl and varying amounts of the calcium salts. No further additions were made. With three parts $CaCO_3$, the maize meal

diet is the rachitogenic diet 2965 of Steenbock & Black [1925] and is that normally used in this laboratory for producing rickets.

Examination of bones. The knee joints of the rats were X-rayed at the end of each experiment and in some instances during the course of the experiment. The femurs were removed on the day the rats were killed. They were cleaned by hand and extracted with ethanol in a Soxhlet apparatus, dried, weighed and ashed at dull-red heat. The results are expressed as the percentage of ash in the dried bones.

RESULTS

Effect of calcium carbonate and of calcium phosphate on the ash content of bones

(*a*) *White flour.* With diets containing no added Ca, the ash content of the bones was, in three experiments, between 32 and 35% (Fig. 1). With added $CaHPO_4$, the maximal ash content of about 50% was reached with approximately 0·4% Ca and little difference was produced with an increase of Ca up to 3%. With added $CaCO_3$, however, the ash content never exceeded 42%, a level reached with 0·2% Ca; higher levels of $CaCO_3$ reduced the ash content, which eventually fell to 28%, i.e. below that of the animals receiving no added Ca. In a preliminary experiment the rats received a supplement of aneurin only and no riboflavin; although this influenced their growth rate (see below), it did not affect the ash content of the bones.

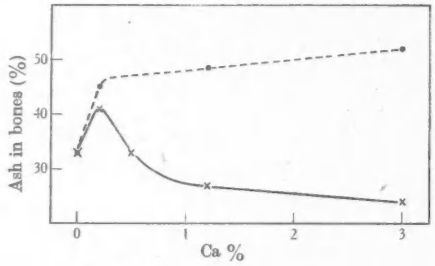

Fig. 1. White-flour diet: effect of $CaCO_3$ (continuous line) and $CaHPO_4$ (dotted line) on bone ash.

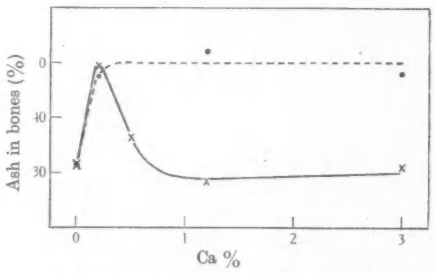

Fig. 2. Wheatmeal diet: effect of $CaCO_3$ and $CaHPO_4$ on bone ash.

(*b*) *Wheatmeal flour.* In many respects, the experiments with wheatmeal flour (Fig. 2) gave results similar to those with white flour. The bones of animals on the basal diet with no added Ca had an ash content of about 32%. The addition of $CaHPO_4$ to the diet produced an ash content of about 50% with some 0·4% Ca, and there was no change with an increasing amount of Ca. Addition of $CaCO_3$ resulted in a maximal ash content at about 0·2% Ca, which rapidly fell with increasing amounts of dietary Ca to a value slightly below that of the animals on the basal diet. The only difference between the results with the white and with the wheatmeal flours appeared to be that the maximal calcification produced by the addition of $CaCO_3$ to wheatmeal flour was greater (about 50%) than that produced with white flour (about 42%); that is, with the former the maximal ash content was the same whether $CaCO_3$ or $CaHPO_4$ was added. Since, however, a slight difference in the amount of dietary $CaCO_3$ in the region of 0·2% Ca produced such a great effect in the bones it is possible that an addition to white flour slightly higher or lower than this might also have resulted in an ash content approaching 50%, and the apparent difference may be fortuitous.

(*c*) *Maize diet.* The results with the maize-gluten mixture were similar to those with flour (Fig. 3).

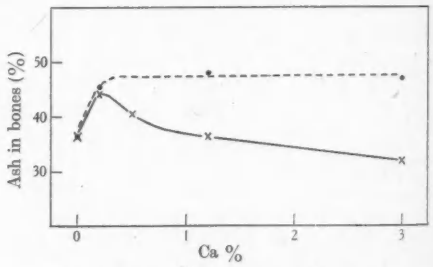

Fig. 3. Maize-gluten diet: effect of $CaCO_3$ and $CaHPO_4$ on bone ash.

The addition of $CaHPO_4$ in any concentration above 0·4% Ca produced a high degree of calcification. Lower concentrations (0·2% Ca) of $CaCO_3$ produced an improvement in calcification, but this deteriorated with higher concentrations. This diminution in effect was not so rapid as with the flour diets; for example, the ash content of the flour diets had reached its minimal level with 1·2% Ca, whereas with the maize diet there was a further decrease from 37% with 1·2% Ca, to 32% with 3% Ca.

Effect of calcium carbonate and calcium phosphate on growth

It is known that, other things being equal, the degree of rickets which an animal develops is proportional to its rate of growth. It might have been thought that the differences in the calcification noted above were due to differences in rates of growth; the higher levels of dietary $CaCO_3$, for example, might be producing their rachitogenic effect by increasing the rate of growth. Such an explanation, however, must be discounted. A study of the growth curves shows that the rate of growth was lowered in each instance by the higher amounts of $CaCO_3$ in the diet (Figs. 4–6). Also the rate of growth on the flour diets was less with $CaCO_3$ than with the $CaHPO_4$, whilst the reverse was true with the maize-gluten diet; as we have seen, in all of these diets the phosphate at high levels of intake produced better calcification than the carbonate.

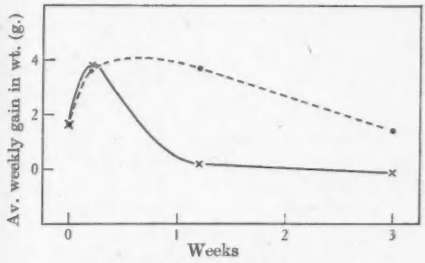

Fig. 4. White-flour diet: effect of $CaCO_3$ and $CaHPO_4$ on growth.

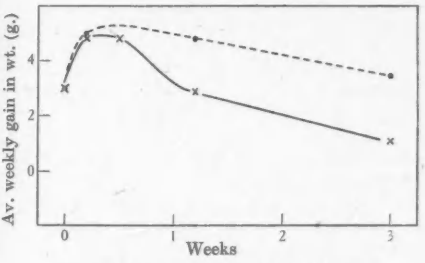

Fig. 5. Wheatmeal diet: effect of $CaCO_3$ and $CaHPO_4$ on growth.

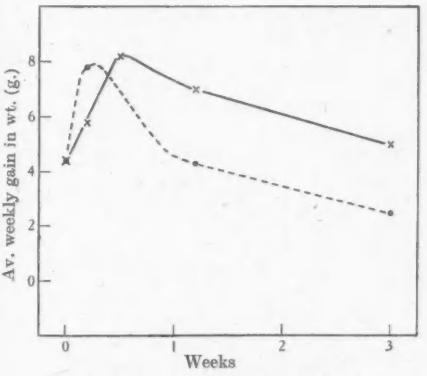

Fig. 6. Maize-gluten diet: effect of $CaCO_3$ and $CaHPO_4$ on growth.

There are other interesting features of the growth curves which are worth noting. Growth on the flour diets to some extent paralleled the ash content of the bones; this was especially noticeable with the white-flour diets containing $CaCO_3$. It was less true with those containing $CaHPO_4$, where the higher levels of this salt diminished growth, although they did not decrease the ash content of the bones. With the maize-gluten diet there was less parallelism; for example, with higher amounts of Ca, the $CaHPO_4$ resulted in a lower growth rate than $CaCO_3$, although it produced better calcification. In all instances growth was better on the wheatmeal-flour diet than on the white-flour diet, even though the latter contained added aneurin and riboflavin [cf. Chick, 1940]. In the preliminary experiments, to which reference has already been made, in which aneurin only was given, there was, in the absence of added Ca, a slight average loss of weight (0·7 g./week) compared with a gain of 1·7 g. when both vitamins were given. When 0·2% Ca or more was added, either as $CaHPO_4$ or as $CaCO_3$, the growth-promoting effect of riboflavin was not observed.

DISCUSSION

It is known that the calcification of bone, especially in the absence of vitamin D, is to a large extent dependent on the Ca/P ratio in the diets. The different effects of the addition of $CaCO_3$ and $CaHPO_4$ to the diet on the bones of the animals appear to be due to this cause. In the absence of added Ca the ratio Ca/P is always low; 0·16 with white flour (70% extraction), 0·10 with wheatmeal flour (unfortified; 85% extraction) and 0·10 with the maize-gluten diet (Tables 1, 2). Addition of $CaHPO_4$ increases the ratio, which reaches a value of one, or slightly more, for all the diets, with an addition of 1·2% Ca as $CaHPO_4$, and increases only slightly with further additions. With additions of $CaCO_3$ the ratio increases to very much higher levels. With 0·2% Ca as $CaCO_3$, the ratio is 2·05 for white flour, 1·10 for wheatmeal flour and 1·00 for the maize-gluten diet; with higher levels of $CaCO_3$ the ratio rises rapidly, so that with 1·2% Ca it is between 5 and 10 and with 3% Ca it is between 13 and 28. In all instances, therefore, maximal calcification occurred with a ratio between 1 : 1 and 2 : 1.

Table 1. Ca *and* P *content of flours and maize-gluten mixture*

	Ca (mg./100 g.)	P (mg./100 mg.)	Ca/P
White flour	16·7	106	0·16
Wheatmeal flour	20	200	0·10
Maize-gluten mixture	22·3	221	0·10

These values have been very kindly supplied by Miss E. M. Widdowson. The values for the white flour and the maize-gluten mixture were from actual analyses of the samples used in these experiments; the values for the wheatmeal flour were average values obtained from the analysis of a large number of samples, in which it was found that the range of variation was fairly small.

Table 2. Ca/P *ratios of flours and maize-gluten mixture with various additions of* $CaCO_3$ *and* $CaHPO_4$

Addition to diet (g./100 g.)	Ca/P: White flour	Wheatmeal flour	Maize-gluten mixture
0	0·16	0·10	0·10
$CaCO_3$ 0·05	0·64	0·35	0·33
0·2	2·05	1·10	1·00
0·5	4·87	2·60	2·36
1·2	11·5	6·10	5·54
3·0	28·5	15·1	13·7
$CaHPO_4$ 0·05	0·46	0·29	0·28
0·2	0·83	0·62	0·59
1·2	1·17	1·08	1·06
3·0	1·29	1·20	1·18

Adequate calcification depends not only on the ratio Ca/P but also on the absolute amounts of Ca and P; if both are low, the bones will not be well calcified. It may be that the poor calcification which occurred on the basal diets without added Ca was due to this fact and not to the very low Ca/P ratios. The amounts of P in the wheatmeal bread and maize-gluten diets are, however, fairly high. It seems likely therefore that the low ash content produced by the basal diets represents rickets of the 'low-calcium' type, whilst the low ash content produced by the higher levels of $CaCO_3$ represents rickets of the 'low-phosphorus' type. The mechanism of the production of 'low-phosphorus' rickets with high Ca/P ratios seems to be a reduction of the absorption of P by the excess of Ca.

It is recognized that the metabolism of Ca and P in human beings is appreciably different from that in rats. For example, rats usually absorb nearly 100 % of their dietary Ca, a proportion much higher than that ever reached in human subjects. Nevertheless, a comparison of rickets in human beings and in rats is not without some value. In both there is excessive faecal loss of $PO_4^{\equiv}$ and sometimes of Ca, as well as a low blood $PO_4^{\equiv}$. Administration of vitamin D in both cases restores these values to normal.

That $CaCO_3$ might be rachitogenic in human beings as well as in rats is suggested by experimental observations. McCance & Widdowson [1942] have demonstrated that absorption of $PO_4^{\equiv}$ in experimental subjects is lowered by the inclusion of $CaCO_3$ in bread. In experiments on babies and children, Stearns & Jeans [1934] showed that Ca and P were adequately retained in the body when given as milk or as $CaHPO_4$ but less was retained when Ca was given as carbonate, gluconate or lactate. Only one study, however, was made with $CaCO_3$.

The quantity of $CaCO_3$ at present added to wheatmeal bread is 65 mg. Ca to 100 g. flour (i.e. 0·065 %). In our experiments with rats these small amounts always result in improved calcification. It would, however, be unwise to transfer these quantitative results directly to the question of human rickets. Moreover, the rats were receiving diets restricted to a degree which would certainly not obtain in human subjects. The work here reported may best be considered as tests of the relative value of $CaCO_3$ and $CaHPO_4$ in favouring bone formation, using rats as a 'biological indicator'. The results suggest that at high levels of dosage the carbonate is inferior to the phosphate. At lower levels, comparable with the quantities added to bread, $CaCO_3$ is as efficacious as $CaHPO_4$ in improving the calcification of the bone of the rat. Whether the carbonate is as good as the phosphate for children, as obtains with growing rats, in the quantities likely to be ingested, can be determined only by trial.

SUMMARY

1. The effect has been studied in rats of the addition of calcium carbonate or calcium monohydrogen phosphate to basal diets consisting of white flour, wheatmeal flour or a maize-gluten mixture.

2. In all instances the addition to the diet of carbonate increased the ash content of the bones when it was added in small amounts in the proportion present in National Wheatmeal bread; larger amounts, however, led to a reduction in the ash content. Phosphate, on the other hand, produced adequate calcification, not only in these small amounts, but also in amounts up to 3 % Ca.

3. These differences were not due to the known aggravation of rickets which results from an acceleration of growth. With the white-flour diet, for example, the gain in weight was less with added carbonate than with phosphate.

4. The applicability of these results to human dietaries is discussed.

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The Rate of Enzymic Hydrolysis of Phosphoric Esters

2. RELATION OF STRUCTURE TO DISSOCIATION CONSTANT, MICHAELIS CONSTANT, AND RATE OF HYDROLYSIS

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Since Fischer [1898] suggested the possibility that a chemical combination took place between an enzyme and its substrate, the kinetics of many enzyme reactions have been explained by postulating such a combination and applying the mass action laws. The classical example of this form of treatment is the equation of Michaelis & Menten [1913]. In their study of mammary gland phosphatase, Folley & Kay [1935] obtained results which were in good agreement with the requirements of this equation, when allowance was made by the method of Haldane [1930] for inhibition with high substrate concentrations. Martland & Robison [1927] found no variation of activity of phosphatase with concentration, but our results suggest that these workers were using concentrations of substrate too high to show the Michaelis-Menten effect.

Several workers have noted the different rates at which phosphoric esters are hydrolyzed by phosphatase, e.g. Martland, Hansman & Robison [1924], Kay [1926; 1932] and Asakawa [1928]. King & Armstrong [1934] showed that hydrolysis of phenyl phosphate by bone phosphatase is more than twice as fast as that of glycerophosphate and many times faster than that of ethyl phosphate. King & Delory [1939] studied other phosphoric esters and suggested that the hydrolysis rates might be related to the Michaelis-Menten constants. In the present paper we have made a study of the rates of hydrolysis, optimum pH, and Michaelis constants, together with determination of the degree of dissociation of the phosphoric esters employed. The following esters were used: α-glycero-, β-glycero-, ethyl-, phenyl-, *o*-bromophenyl-, *m*-bromophenyl-, *p*-bromophenyl-, 2:4-dibromophenyl-, 2:4:6-tribromophenyl-, *p*-nitrophenyl-, and *o*-cresyl phosphates.

METHODS

Preparation of a potent phosphatase. A potent preparation was desirable in order to minimize the effect of contaminants and to permit short (5 min.) periods of hydrolysis, during which the rates of enzymic hydrolysis were not very different from initial velocities. Dialyzed and precipitated kidney and intestinal preparations were not effective. The potent phosphatase derived from dog faeces by the method of Armstrong [1935] was used. The brown powder obtained by procedures 'A' and 'B' of this author was dried *in vacuo*. In the presence of an optimal amount of Mg it showed an activity of 10,000 King-Armstrong phosphatase units/g., and still retained its activity after 5 years in the refrigerator.

A solution of 1 unit/4 ml. was prepared as follows: 1 mg. of the preparation was dissolved in 30 ml. 0·9% NaCl containing enough $MgSO_4$ to make the final $MgSO_4$ concentration $M/100$. The phosphatase activity (about 30 units/100 ml.) was measured accurately by the method of King, Haslewood & Delory [1937]. The solution was then diluted so as to contain exactly 25 units/100 ml.

Preparation of phosphoric esters, see King & Nicholson [1939].

Determination of degree of dissociation of phosphoric esters. The apparatus used for the pH estimations was a hydrogen electrode with saturated calomel electrode and the Moloney [1921] platinum electrode. The latter was designed to hold a film of the solution, whose pH is to be determined, across the platinum electrode. Since it was only necessary for a small volume of solution to become saturated with H_2, ionic equilibrium was reached immediately. The potentiometer available was accurate to the nearest 0·01 pH. The apparatus was checked with a standard acetic acid-sodium acetate buffer of pH 4·63. The following procedure was adopted: 20 ml. of a $N/10$ solution of the salt of the phosphoric ester were pipetted into the reaction vessel. $N/10$ HCl was added in 0·2 ml. quantities, the pH being determined after each addition, and the pK determined graphically [cf. Pirie & Pinhey, 1929].

The graphs for *p*-bromophenyl-, *o*-bromophenyl-, phenyl- and α-glycero-phosphate are given as examples in Fig. 1. The pK's are included in Table 1.

Choice of a suitable buffer system. A buffer system covering the range of pH 7–11 and having the minimum effect on the rate of hydrolysis was obtained by combining the Michaelis [1930] veronal—HCl buffer and the Kolthoff [1925] Na_2CO_3-HCl buffer [King & Delory, 1940].

Determination of the optimum pH *for hydrolysis and rate of enzyme activity.* 4 ml. phosphatase solution (1 unit), 4 ml. buffer and 2 ml. $M/100$ substrate were incubated at 37° for exactly 5 min. The enzyme action was stopped by adding 2 ml. acid molybdate [King & Delory, 1939].

Determination of Michaelis constant:

$$\frac{v}{V}=\frac{x}{K_m+x}, \quad \text{or} \quad \log\frac{1}{x}=\log\frac{1}{K_m}+\log\left(\frac{V}{v}-1\right),$$

where x is the molar concentration of substrate, v is the velocity of hydrolysis, K_m is the dissociation constant of the enzyme-substrate compound (Michaelis constant) and V is the velocity when x is large compared with K_m. A curve of this equation will clearly be of the form of the dissociation of a weak acid and at the point at which $v/V=\frac{1}{2}$, i.e. where the velocity of hydrolysis is half the limiting velocity, K_m will be equal to the substrate concentration x.

Two ml. of the appropriate buffer solution together with an appropriate amount of water to make a final total volume of 10 ml. were pipetted into 15 ml. volumetric flasks. The flasks were placed in a water bath at 37° for 2 min. to allow the contents to attain the temperature of the bath. Two ml. of the enzyme solution were added, followed by the stated amount of $M/100$ substrate solution. The arrangement for the 7 flasks used was as follows:

Flask	ml. $M/100$ substrate	ml. water	log molarity of substrate
1	0·1	5·9	−4·00
2	0·2	5·8	−3·70
3	0·5	5·5	−3·30
4	1·0	5·0	−3·00
5	2·0	4·0	−2·70
6	4·0	2·0	−2·40
7	6·0	—	−2·23

After exactly 5 min., 2 ml. acid molybdate were added. An observer stood by with a stop-watch to note the time at which the substrate was added to each flask, and to signal when the enzyme activity should be stopped by the addition of the molybdate solution. 0·5 ml. aminonaphthol-sulphonic acid was then added to each flask, and water to the mark [King, 1932]. The amount of inorganic P liberated was plotted against the logarithm of the substrate concentration. Inspection of the graph gave the substrate concentration at which half the limiting velocity was reached and the value was checked by substituting for several points in the Michaelis-Menten equation.

RESULTS

In Table 1 are given for each ester: (*a*) the negative logarithm of the dissociation constant (pK); (*b*) the pH at which hydrolysis by the faecal phosphatase

Table 1. *Rates of enzymic hydrolysis of phosphoric esters*

Phosphoric ester	pK	Optimum pH	Inorganic P enzymically liberated in 5 min. (mg./unit phosphatase)	K_m
p-bromophenyl	5·44	9·96	0·099	0·0003
2:4-dibromophenyl	5·60	9·95	0·085	0·0004
o-bromophenyl	5·60	9·80	0·086	0·0004
p-nitrophenyl	5·70	9·80	0·082	0·0005
phenyl	5·73	9·76	0·069	0·0006
o-cresyl	6·04	9·55	0·042	0·0008
2:4:6-tribromophenyl	6·10	8·94	0·036	0·0008
β-glycero	6·34	8·82	0·029	0·0012
α-glycero	6·44	8·62	0·019	0·0014
ethyl	6·45	8·08	0·008	0·0025
m-bromophenyl	Almost undissociated		Very little hydrolyzed	—

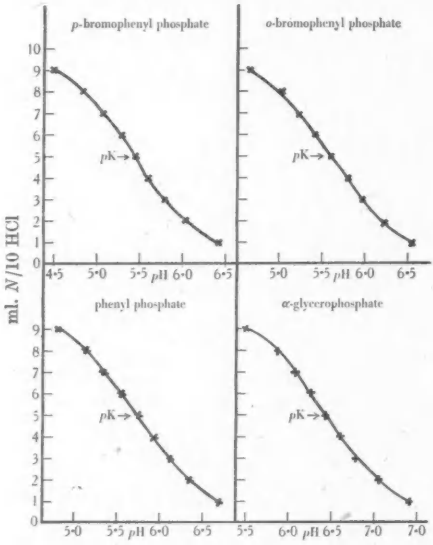

Fig. 1.

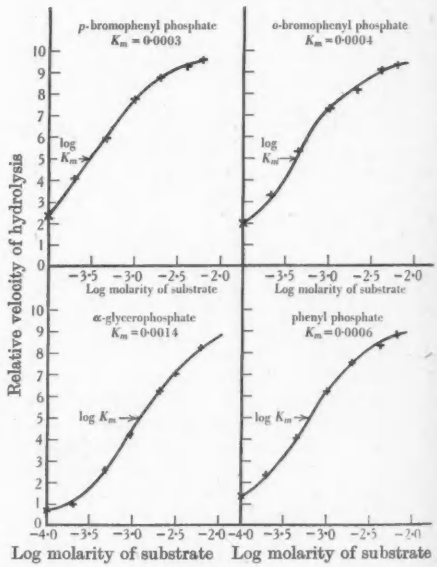

Fig. 2.

Fig. 1. Dissociation curves for phosphoric esters. 20 ml. of a $N/10$ solution of the salt of the ester titrated with $N/10$ HCl. Continuous lines were drawn from data derived from the equation $\mathrm{pH} = pK + \log \alpha/(1-\alpha)$, the value of the constant pK being chosen so as to give the best fit for the determined values.

Fig. 2. Curves showing Michaelis effect of phosphoric esters and faecal phosphatase at the optimum pH. The continuous lines were drawn from data derived from the equation $\log \frac{1}{x} = \log \frac{1}{K_m} + \log\left(\frac{V}{v} - 1\right)$, the values of the constant K_m being chosen so as to give the best fit for the determined values.

occurs at the maximum rate (optimum pH); and (c) the Michaelis constant (K_m) for the enzymic hydrolysis at the optimum pH. The pH-dissociation curves for *p*-bromophenyl-, *o*-bromophenyl-, phenyl- and α-glycero-phosphate are given in Fig. 1, while the curves showing the rate of enzymic hydrolysis at the optimum pH for the same four esters are illustrated in Fig. 2. In the latter case, the method adopted by Folley & Kay [1935] has been followed in the presentation of the graphs, the continuous line showing the theoretical curves for the Michaelis constant specified, and the experimental values being represented by crosses.

A preliminary experiment had shown that in the case of phenyl phosphate the theory of Michaelis was closely followed up to a concentration of 0·009 *M*, but that above this concentration, the velocity of hydrolysis decreased in accordance with the theory of Haldane [1930]. In the main series of experiments the higher concentrations, where deviations from the Michaelis-Menten equation might have been expected, were not investigated, since the main object was the determination of the Michaelis constants for the several esters.

The results indicate that with increasing acidity (i.e. decreasing *pK*) of the substrate there is an increase in the amount of hydrolysis, whilst the pH of optimum hydrolysis shifts to an increasingly alkaline reaction.

DISCUSSION

All the aromatic phosphoric esters (except *m*-bromophenyl) are more acidic and combine more strongly with the enzyme than the aliphatic esters. Of the latter ethyl phosphate is a weaker acid and is less strongly attacked enzymatically than the glycero-phosphates, which recalls the fact that ethyl chloride is much less chemically active than the chlorohydrins. The addition of an aliphatic group to an aromatic nucleus, as in *o*-cresyl phosphate, appreciably lowers activity.

The difference in behaviour of the *o*-, *m*-, and *p*-phenyl phosphates is noteworthy. The *p*-compound is the most rapidly hydrolyzed, whilst the *o*-compound occupies an intermediate position. These observations may be compared with the facts that *p*-methoxybenzyl bromide is hydrolyzed by acids much more rapidly than the benzyl bromide. The *o*-compound is also hydrolyzed more rapidly than the parent compound, whilst *m*-methoxybenzyl bromide is hydrolyzed less rapidly than the unsubstituted benzyl bromide [Waters, 1935]. It is also quite common to find a descending order of acidity in passing from *p*-compounds through *o*- to *m*-. An example may be given from the *pK*'s of the nitrophenol isomers: *p*-nitrophenol 7·15, *o*-nitrophenol 7·17, *m*-nitrophenol 8·41.

Martland & Robison [1927] suggested two possibilities: (1) that phosphatase had the properties of a weak base whose undissociated molecules combine with a phosphoric ester to form a compound, which thereupon breaks down into the products of hydrolysis, or (2) that the enzyme combined with the ester to form a compound having the properties of a weak acid, whose anions suffer spontaneous decomposition. Our results seem to favour the first hypothesis, since an enzyme having basic properties might be expected to have the greatest affinity for that substrate with the most electro-negative grouping. Thus the greater the affinity between the substrate and the enzyme, the greater will be the rate of enzymic activity and the smaller the Michaelis constant.

Many workers, e.g. Martland & Robison [1927], have shown the gradual inactivation of phosphatase in alkaline solution. Kay [1932] has suggested that the rate of hydrolysis continues to increase with rise of alkalinity, and the apparent optimum is set by the increasing rate of enzyme destruction. This accords well with the further observation of Robison, who found that the enzymic hydrolysis of glycero-phosphate reached a maximum at pH 8·4 for an 18 hr. period of hydrolysis, and 9·4 for shorter periods. It is therefore suggested that the higher pH optimum values obtained here for the more acid esters are due to a greater protection of the enzyme against the inactivating effect of the hydroxyl ion. There are many examples of the protection of an enzyme by its substrate, e.g. trypsin is protected against heat inactivation by egg albumin, malt amylase by starch, and urea protects urease against the inactivating effect of certain dyestuffs.

The following explanation may, therefore, account for the results described in this communication. The enzyme has basic properties which enable it to combine with the substrate, the affinity between the two substances increasing with decreasing *pK* of the substrate. The greater this affinity, in other words, the smaller the Michaelis constant, the greater is the rate of hydrolysis, the greater is the degree of protection given to the enzyme against the inactivation effect of hydroxyl ions, and in consequence the higher the optimum pH.

SUMMARY

1. The rates of hydrolysis of several phosphoric esters by faecal phosphatase have been investigated, together with the Michaelis constants of the reactions and the dissociation constants of the esters.

2. The results show that with decreasing *pK* of the substrate the rate of hydrolysis increases progressively, and the enzyme is optimally active at a more alkaline pH. The Michaelis constants decrease with decreasing *pK* of the substrate.

3. These observations are consistent with the hypothesis of Martland & Robison [1927] that the enzyme possesses the properties of a weak base, whose undissociated molecule combines with the substrate to form a compound which breaks down into the products of hydrolysis, and with the postulate of Kay [1932] that the rate of hydrolysis continues to increase with increasing alkalinity and that the apparent optimum is set by the increasing rate of enzyme destruction.

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The Nutritive Value of Potato Protein for the Pig

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It has long been known that only about one-half of the nitrogen of the potato is in the form of protein, the remainder being in amino-acids and amides, chiefly asparagine. Neuberger & Sanger [1942] have recently shown that not only the total N of the potato, but also the relative proportions of the above three types of nitrogenous compounds, are widely different in different varieties, and that generally the amount of true protein N is rather less than half the total.

Although it is common convention in agricultural practice to reckon the protein value of the potato N as that obtained by adding together the true protein value and one-half of the value obtained by multiplying the non-protein N by 6·25, in this paper the proportion of the N in the different diets, rather than that of the protein, is considered, and the term crude protein is used to indicate the figure obtained by multiplying the total N by 6·25.

The maintenance of N equilibrium in adult man by potatoes was established by Hindhede [1913], Abderhalden, Ewald, Fodor & Rose [1915], Rose & Cooper [1917], Rubner & Thomas [1918] and by Kon & Klein [1928]. The last-named workers found that N equilibrium and good health could be maintained for several months in an adult man and woman on a diet of steamed potato, butter fat and fruit. The daily N intakes averaged 5·7 and 3·8 g. respectively.

The value of potato nitrogen for the support of growth. The value of potato N for the support of growth is not so clear, for it is difficult to obtain a sufficiency for young animals with potato alone since the protein is diluted with so much starch. McCollum, Simmonds & Parsons [1918] found that hardly any weight increase occurred in young rats when potatoes provided the only source of N. The diets contained but 7–8% crude protein. However, growth was no better when cereal proteins were fed at the same level but was much improved on addition of 5 or 10% of casein. Growth of rats on diets containing 9% protein from oats, maize, peas or millet was much better and reproduction occurred when a proportion of these was substituted by potato, thus indicating a supplementary action of the N of the latter for that of the above grains [McCollum, Simmonds & Parsons, 1921]. Hartwell [1925; 1927] found that potato providing 7·6% crude protein in the diet supported only very poor growth in rats and was quite insufficient for lactation.

Mitchell [1924*b*] determined the biological value of potato N for growth and maintenance in young rats by his modification [1924*a*] of Thomas's N balance technique. The biological value, when the diet contained 5% crude protein, was 68·5, as compared with 72 for maize, 78·6 for oat, and 86·1 for rice protein. With 10% crude protein in the diet, the biological values were 66·7 for potatoes, 59·6 for maize

and 64·9 for oats. These results are somewhat inconclusive, since potatoes appeared to be inferior to cereal proteins when the diet contained 5% crude protein but as good or better when it contained 10%.

Schiftan [1933] made a series of careful N balance experiments with pigs fed on diets in which respectively potatoes, barley and other foods were the only sources of protein. The tests were made with protein given at maintenance level, and also at a level to support both maintenance and growth. His experiments were carried out on four animals only and the collection periods were of 5 days' duration; his technique was, however, unexceptionable and good agreement was obtained in duplicate tests made on different pigs. For maintenance, the biological value of potato N in a diet containing 2·2% crude protein was 69·6, and that of barley N in a diet containing 2·3% was 58·7. Milk, casein (purified) and fish meal gave figures of 100·6, 69·3 and 66·2 respectively. For growth and maintenance, the biological value of potato N was 73·2 in a diet containing 13% protein. The value for N of fishmeal was of about the same order and the values for that of milk and casein again superior. Potato N was thus slightly superior to that of barley in the tests at both low and high levels of intake. Since biological values of proteins normally fall as protein level is raised, one would expect that the value obtained for barley for growth and maintenance in the above experiment would be relatively too low in comparison with that given for potato. However, Schiftan, contrary to other investigators, found in general that his biological values increased slightly with level of protein fed.

Jones & Nelson [1931] obtained poor growth with rats on diets containing 9% of crude protein from potato. Neither addition of gelatin, nor a supplement of tryptophan or of tyrosine and cystine with or without gelatin, improved matters, but addition of casein or lactalbumin supplemented the potato, and good growth occurred. The potato N was fed as a concentrate, prepared by removal of the starch, and contained 90% of the original N. From this experiment it appeared that the potato lacked some unidentified essential amino-acid. Beadles, Braman & Mitchell [1930], on the other hand, also working with rats, had previously found that cystine itself improved growth when added to a potato diet containing 8–9% crude protein. The potatoes were boiled and dried at low temperature.

The biological value of the true protein of potato appears to be fairly high. Kon [1928], using Mitchell's N balance technique for estimating its value for growth and maintenance of rats, found a biological value of 71 for tuberin compared with 68 for caseinogen; both proteins were fed at a level of 8·5–8·6% in the diet. By the method of Osborne, Mendel & Ferry [1919] with growing rats, he obtained a maximum figure of 2·0 g. weight increase per g. of protein intake, when the diet contained 8% protein.

Mangold & Columbus [1937] used Schiftan's technique to determine the biological value, for maintenance of N balance in pigs, of two dried potato protein concentrates called 'potato protein pulp flakes' (*Kartoffeleiweiss-pulpe-flocken*) and 'potato protein flakes' (*Kartoffeleiweiss-flocken*), respectively. In these preparations nearly all the N was in the form of true protein. The biological value of the 'potato protein pulp flakes' was 86·3 in a diet containing 3·8% protein and 88·5 in one containing 7·1%; that of the 'potato protein flakes' was 83·3 in a diet containing 5·1% protein and 79·0 when it contained 9·7%.

Digestibility of potato nitrogen. In the following paragraphs the coefficient of digestibility refers to 'apparent' digestibility, the figure for which is calculated from the difference between the N ingested and that eliminated in the faeces, on the assumption that the latter is derived from undigested food. The faeces, however, also contain N of endogenous origin, e.g. that contributed from gastric and intestinal secretions from, and into, the gut. A large proportion of the N of faeces is contained in its bacterial component and it is uncertain whether these bacteria are nourished from endogenous nitrogenous substances or from those of undigested food. The true digestibility of any food will therefore always be greater than the apparent digestibility.

The figures quoted in the literature for the digestibility of the crude protein of potatoes are very variable and usually low. Table 1 gives a summary of the figures obtained with swine for potatoes and various potato products; it will be seen that the coefficients of apparent digestibility vary from 0 to 84%! There are four possible explanations of this great variation: (1) that the variations are due to differences in the samples or varieties of potatoes before cooking or processing, (2) that the digestibility is affected by processing, (3) that it depends on the bacterial flora inhabiting the gut, (4) that it is affected by other constituents of the ration. There is not sufficient evidence in the literature to permit discussion of the first and third possibilities, but so far as the effect of processing is concerned, it is noticeable that all the lower figures for digestibility with the pig have been obtained with potatoes dried in various ways. With herbivora, on the other hand, comparatively low digestibility coefficients have frequently been obtained for cooked fresh potatoes. For example, Woods [1919] found a coefficient of 43·4% for the sheep, Brüggemann [1939] 58·3% for the rabbit, and Woodward, Davidson, Watson, Robinson & Muir [1938] 65·0% for the steer. Kellner, Just, Eisenkolbe & Poppe [1908] determined the digestibility of different dried potato products, fed in combination with soya-bean meal, with pigs and sheep; with both there were large differences between the different products, but the order of their merit was different in the two species. For example, potato flakes dried by the Pauckseh process gave the highest coefficient (75·5%) with pigs and the lowest (14·4%) with sheep.

It is probable that some of the very low figures may be connected with the method of computation. In many investigations determination was first made of the digestibility of a ration, often of mixed foodstuffs, and then again when a portion had been substituted by potato or potato products. Any lowering in digestibility in the second case compared with the first was then attributed solely to the potato. If the potato was present in relatively small proportion, the result of such a calculation will give an unduly low figure for its digestibility.

Fingerling [1932] obtained the very low digestibility coefficient of 29·2% for the crude protein of potato cossettes when fed to pigs with fishmeal and barley meal. When the basal ration consisted of fishmeal and potato flakes, the digestibility of the nitrogen of additional potato flakes, however, was 70·5%. It is possible that in the first test the digestibility of the fishmeal and barley meal was depressed by addition of the potato cossettes and that, in the second, the addition of extra potato flakes caused no further depression in the digestibility of a ration which already contained them. With steers, the digestibility of the crude protein in both flakes and cossettes was low, but the order was reversed; the coefficient for flakes was negative while that for the cossettes was 30·9%; in these experiments the

Table 1. *'Apparent' digestibility of the crude protein of potatoes for pigs*

Type of potato product	Digestibility coefficient of crude protein	Author
Raw	84·5	Snyder [1895]:
Cooked	82·0	"
Dried 'Kartoffelscheiben'	53·9	Kellner *et al.* [1908]
Dried 'Kartoffelschnitzel' (Schütz)	46·2	"
Dried 'Kartoffelschnitzel' (Knauer)	63·5	"
Dried 'Kartoffelschnitzel' (Petry & Kecking)	70·6	"
Dried 'Kartoffelschnitzel' (Venuleth & Ellenberger)	42·9	"
Dried 'Kartoffelflocken' (Paucksch)	75·5	"
Dried 'Presskartoffel' (1)	26·7	Kellner & Neumann [1910]
Dried 'Presskartoffel' (2)	<0·0	"
Dried 'Kartoffelflocken'	70·5	Fingerling [1932]
Dried 'Kartoffelschnitzel'	29·2	"
Cooked	56·5*	Schiftan [1933]
Cooked	67·3*	"
Dried 'Kartoffeltrockensaft-Pülpeflocken'	62·3	Nehring & Schramm [1937]
Dried 'Kartoffeleiweissflocken'	73·9	Mangold & Columbus [1937]
Dried 'Kartoffeleiweiss-Pulpe-Flocken'	66·5	"
Cossette meal	39·9	Woodman & Evans [1939]

* True digestibility.

basal ration was composed of wheat straw, 'molasses slices' and cotton-seed meal. Fingerling notes in his paper that in the case of the steers the addition of potato products appeared to depress the digestibility of the N and N-free extract of the basal ration.

Woodman & Evans [1939] found the digestibility of the crude protein of potato cossettes, ground into a meal for pigs and sheep, to be 45·4% for the sheep and 39·9% for the pig. Tests *in vitro* gave a figure of 94%. Here again it is possible that the apparent digestibility of the barley meal, weatings and white fish meal in the basal ration may have been lowered when fed with the potato cossette meal and thus a low estimate made of the digestibility of the latter.

The experiments of Kellner & Neumann [1910], on the other hand, gave little evidence that depression of the digestibility of the N of the constituents of the ration was caused by the presence of potato. They determined the digestibility of pressed dried potatoes, first when fed alone and later when fed with soya-bean meal. The coefficient of digestibility of the N of the potatoes fed alone was 26·7%. Using this coefficient they calculated from the second experiment a coefficient for soya-bean meal of 94%, a figure which could not have been so high if the pressed dried potatoes had appreciably lowered the digestibility of the soya-bean meal. However, from a further trial with a second sample of dried pressed potatoes, also fed with soya-bean meal, they calculated a negative digestibility for the crude protein of the potatoes when the figure of 94% for the soya bean was used in the computation.

Section 1. GROWTH EXPERIMENTS

METHODS

The object of the experiment was to compare the growth of pigs which received all their protein as barley, or as barley supplemented with certain levels of casein, with that of pigs which received all their protein as potato, or as potato supplemented with identical levels of casein.

Mitchell's paired feeding method was adapted to the conditions of this experimental layout. Twenty-four pigs (17 hogs and 7 gilts) of the Wessex Saddleback breed were obtained from a local farm, and were kept in the same stalls and under the same system of management as those observed by Macrae, El-Sadr & Sellers [1942] in their studies on yeast protein. The animals were from four litters, all of which were farrowed between 6 and 16 January 1941; their individual identity was unknown. During the 3 weeks in which they were housed at the Institute prior to the commencement of the experiment, they were provided with a commercial pig food, with a few potatoes added daily. When they were 15–16 weeks of age, and of 25–36 kg. body weight, the groups and subgroups were selected. First, 12 pairs of as nearly equal live weights as possible were chosen: one pig from each of these pairs was allotted at random to a potato diet and the other to a barley diet, thus forming two groups (potato and barley), each comprising 12 animals. The four subgroups, each comprising three animals chosen at random from the potato group and a corresponding three animals from the barley group, received, respectively, unsupplemented diets, and diets containing 1, 3 and 6% casein. All the pigs were individually fed in separate pens. Each of the 12 pigs receiving the potato diets consumed the same amounts of N and of dry matter as its corresponding barley-fed partner. The barley-fed pigs obtained slightly less digestible energy than the potato-fed animals because the barley was less digestible (see below), but there are not sufficiently reliable comparative figures to show whether this implied a lower intake of net energy under optimal conditions of protein intake.

The quantity of food given was regulated by the appetite of that pig of each pair which consumed the smaller amount: this was in every case the potato-fed pig. By this procedure, the results of the experiment could not be vitiated by

inability of the potato-fed pigs to consume as much of their more bulky ration as the barley-fed animals. It was not practicable to equalize the food intakes of animals receiving different levels of casein, since this would have meant reducing the food intake of all pigs to that of the one which ate least food. Except in so far as the food intake of the barley-fed animals was restricted by paired feeding, the diets were so constituted that the protein supply was the factor limiting growth. Only those pigs which received diets containing the highest proportion of N, viz. 2·4%, chiefly from casein, showed a growth rate approaching that obtained with Wessex Saddlebacks under good commercial conditions (H. A. Parsons, private communication; see Fig. 1).

The composition of the foodstuffs and of the experimental rations are set out in Tables 2, 3 and 4. In order to reduce the bulk of the potato diets, half the potatoes (on a dry matter basis) were given as boiled fresh potatoes and half as commercial potato flakes which were ground into a meal before mixing. Corresponding barley and potato rations were arranged to contain the same quantity of N (see Table 3).

The *cod-liver oil* was of the type used in veterinary practice, and was of guaranteed vitamin potency.

The *salt mixture*, made from commercial materials, contained bone ash 2, $CaCO_3$ 1, NaCl 1 and Fe_2O_3 0·1 parts.

The *yeast extract* was made by the method of Macrae *et al.* [1942], from brewer's yeast kindly supplied by Messrs Greene, King and Sons. The extract, which was protein-free, contained 1·607 mg. N/ml., and the daily dose, given separately with the ration of each pig, contained the equivalent of 30 g. dry yeast.

The *boiled potatoes* were from two batches of 'Majestic' potatoes, obtained locally and stored under straw in a tin hut; there was a good deal of sprouting during the experiment, but the sprouts were rubbed off, and the potatoes were washed before cooking. They were cooked in a cage lowered into a large copper of boiling water. The N content of the first batch was 1·58, and of the second, 1·85%, on a dry matter basis.

The *potato flakes* were obtained from the Farmers' Marketing and Supply Co., Ltd., and were processed at the beginning of March 1941. The processing was as follows: raw potatoes were thoroughly washed and cooked in a pressure steamer for approximately 20 min. The cooked potatoes were then passed through a mashing screw and spread over rollers heated by steam at a pressure of 80 lb./sq. in. The flakes contained 1·507% N on a dry matter basis.

The cooked potatoes were carefully mixed with the dry potato meal, starch, salts, cod-liver oil and water before feeding. It was necessary to feed the rations in a very sloppy state and to titivate the appetites of the animals with additions of water towards the end of each meal. The

Table 2. *Composition of foodstuffs incorporated in the diets*

	Dry matter (%)	Total N content of dry matter (%)	Gross energy (kg. cal./100 g. of dry matter)	True protein N (% of total N)	Non-protein N (% of total N)
Potatoes (Majestic): Batch 1	29·90*	1·570*	406·0	41·1	58·9
Batch 2	28·07†	1·848†	402·7	39·8	60·2
Casein	89·53	15·26	567·9	—	—
Potato flake meal	87·47	1·507	403·0	—	—
Barley meal (1)	84·31‡	1·826‡	418·9	—	—
(2)	85·30§	1·869§	418·9	—	—
Yeast extract	—	1·607 mg./ml.	—	—	—
Starch	87·00	—	422·0	—	—
Cod-liver oil	—	—	945·0	—	—

* Mean of 29 duplicate determinations.
† Mean of 27 duplicate determinations.
‡ Mean of 6 duplicate determinations.
§ Mean of 3 duplicate determinations.

Table 3. *Percentage composition of the diets, calculated on dry weights*

Subgroup	Boiled potatoes	Potato flake meal	Barley meal	Casein	Starch	Salt mixture	Cod-liver oil	N content of dry matter (%)
No casein: Barley	—	—	79·90	—	16·60	2·00	1·50	1·49
Potato	45·25	45·25	—	—	6·00	2·00	1·50	1·49
1% casein: Barley	—	—	79·90	1·00	15·60	2·00	1·50	1·64
Potato	45·25	45·25	—	1·00	5·00	2·00	1·50	1·64
3% casein: Barley	—	—	79·90	3·00	13·60	2·00	1·50	1·95
Potato	45·25	45·25	—	3·00	3·00	2·00	1·50	1·95
6% casein: Barley	—	—	79·90	6·00	10·60	2·00	1·50	2·40
Potato	45·25	45·25	—	6·00	—	2·00	1·50	2·40

In addition pigs received yeast extract equivalent to 30 g. dry yeast daily.

The following materials were utilized in the construction of the rations:

The *barley meal* was ground whole barley from a single batch.

The *casein* was 'Ashless G.L.', described by the manufacturers (Glaxo Laboratories, Ltd.) as a 'self-soured' type, well washed with dilute acetic acid. It contained 15·27% N on a dry matter basis.

The *starch* was purest maize starch, obtained from Messrs Mackean of Paisley; it contained about 0·04% N.

Table 4. *Analysis of diets*

Sub-group	Ration	% casein in diet	g. N/100 g. dry diet contributed by				% of total N contributed by				Total quantities/100 g.			
			Boiled potatoes	Flake potatoes	Barley meal	Casein	Boiled potatoes	Flake potatoes	Barley meal	Casein	N (g.)	Digestible N (g.)	Gross energy (kg. cal.)	Digestible energy (kg. cal.)
1	Barley	0	—	—	1·494	—	—	—	100·0	—	1·494	1·212	418·93	366·61
	Potato	0	0·813	0·681	—	—	54·4	45·6	—	—	1·494	1·242	404·08	382·25
2	Barley	1	—	—	1·494	0·151	—	—	90·8	9·2	1·645	1·341	420·39	358·59
	Potato	1	0·813	0·681	—	0·151	49·4	41·4	—	9·2	1·645	1·348	405·54	382·02
3	Barley	3	—	—	1·494	0·455	—	—	76·7	23·3	1·949	1·651	423·31	366·59
	Potato	3	0·813	0·681	—	0·455	41·8	34·9	—	23·3	1·949	1·671	408·46	385·18
4	Barley	6	—	—	1·494	0·909	—	—	62·2	37·8	2·403	2·048	427·68	360·53
	Potato	6	0·813	0·681	—	0·909	33·9	28·3	—	37·8	2·403	2·142	412·83	389·30

amounts of food left unconsumed by the potato-fed animals were often considerable, and it was then necessary to weigh the residues and to determine their water content.

The analyses of the materials are given in Table 2 and details of the composition of the different diets in Tables 3 and 4.

RESULTS

The experimental rations were fed for a preliminary period of a week before live weight increases were recorded. About three days after this feeding was commenced, scouring was noted in the potato-fed animals of each subgroup. This scouring reappeared at intervals throughout the experiment, but was never severe. Otherwise the pigs remained in good health. The condition of the coat remained good in all animals, and no skin lesions developed. Carriage and behaviour were in most cases normal throughout. Coprophagy was never observed. During the 7th week of the test there was an exceptional heat wave, and all the potato-fed animals went off their feed. Pigs 8, 10 and 12 were once observed to vomit and scoured at this time. In addition, pig 21, a barley-fed animal, went off its feed and vomited once. No stiffness of the legs was noticed until the last fortnight of the experiment, when a few of the heavier pigs were a little stiff. This was probably because bedding material was not provided. The joints of the animals did not show any macroscopic lesions on slaughter.

Fig. 1 gives the composite growth curves of the various subgroups; Table 5 gives details of their food intake and weight increases. In 11 of the 12 pairs of pigs, the barley-fed pig grew better. The total weight increase of the barley-fed pigs during the 12 weeks averaged 29·5 kg., while that of the potato-fed animals was 23·6 kg.; the weight increase per unit N intake and per unit digestible N intake was higher, and food intake and digestibility energy per unit weight increase were lower, in the barley-fed animals (Table 5).

The results of our experiment are not, therefore, in accordance with those of Schiftan, who found the N of fresh potatoes of higher biological value than that of barley. It is possible that the value of the N of the potato flakes used in our experiments was somewhat reduced by heat treatment during processing. The pigs in subgroup 1 which received the potato N without casein supplement, made better gains than those fed on maize without supplement in the experiment of Macrae *et al.* [1942]. The initial weights of the pigs in our experiment were, however, initially greater than those used by Macrae *et al.*, which averaged about 20 kg. They would therefore not require so large a proportion of N in their ration, since, as age and live weight increase, relatively more fat and less muscle are laid down.

A comparison of the figures obtained from the four subgroups, summarized in Table 5, shows that:

Table 5. *Experimental data for individual pigs during whole experimental period*

Sub-group	Casein in diet (%)	Pig No.	Pig Sex	Ration	Body weight: At beginning (kg.)	Body weight: At end (kg.)	Body weight: Increase (kg.)	Body weight: Average weekly increase (kg.)	Total dry food intake (kg.)	Total N intake (kg.)	Wt. increase/ unit intake of N	Wt. increase/ unit intake of digestible N	Food intake/ unit wt. gained	Digestible energy consumed/ unit wt. gain (kg. cal./kg.)
1	0	1	♂	Barley	34·6	69·9	35·3	2·94	128·25	1·893	18·7	22·9	3·63	13,300
		2	♂♂	Potato	34·5	66·4	31·9	2·66	129·83	1·931	16·5	19·9	4·07	15,600
		3	♂♂	Barley	35·8	58·6	22·8	1·90	94·21	1·399	16·3	20·0	4·13	15,100
		4	♂♀	Potato	35·2	51·6	16·4	1·37	87·44	1·416	11·6	13·9	5·33	22,300
		5	♂♀	Barley	32·0	49·0	17·0	1·42	77·83	1·158	14·7	18·1	4·58	16,800
		6	♂	Potato	31·5	42·0	10·5	0·88	79·13	1·174	8·9	10·7	7·54	28,900
		Average barley fed			—	—	25·0	2·1	—	—	16·6	20·3	4·11	15,100
		Average potato fed			—	—	19·6	1·6	—	—	12·3	14·8	5·65	22,300
2	1	13	♂	Barley	36·6	63·4	26·8	2·23	102·22	1·670	16·1	19·7	3·81	13,700
		14	♂♀	Potato	34·5	55·8	21·3	1·78	103·87	1·693	12·6	15·4	4·88	18,700
		15	♂♀	Barley	26·6	43·0	16·4	1·37	74·30	1·223	13·4	16·4	4·53	16,200
		16	♂	Potato	27·2	39·8	12·6	1·05	74·38	1·220	10·3	12·6	5·90	22,600
		17	♂♂	Barley	32·0	64·8	32·8	2·73	118·66	1·931	17·0	20·8	3·62	13,000
		18	♂♂	Potato	33·0	61·1	28·1	2·34	120·55	1·963	14·3	17·5	4·29	16,500
		Average barley fed			—	—	25·3	2·1	—	—	15·5	19·0	3·99	14,300
		Average potato fed			—	—	20·7	1·7	—	—	12·4	15·2	5·02	19,300
3	3	7	♀	Barley	33·8	65·2	31·4	2·62	93·14	1·808	17·4	20·5	2·97	10,900
		8	♂♂	Potato	34·6	54·7	20·1	1·68	95·10	1·835	11·0	12·8	4·73	18,300
		9	♂♂	Barley	32·4	60·6	28·2	2·35	95·73	1·859	15·2	17·9	3·39	12,400
		10	♂♀	Potato	32·7	54·2	21·5	1·79	92·54	1·781	12·1	14·1	4·30	16,600
		11	♂♂	Barley	34·6	70·8	36·2	3·02	114·81	2·219	16·3	19·3	3·17	11,600
		12	♂	Potato	33·6	59·7	26·1	2·18	116·22	2·231	11·7	13·7	4·45	17,200
		Average barley fed			—	—	31·9	2·7	—	—	16·3	19·2	3·18	11,600
		Average potato fed			—	—	22·6	1·9	—	—	11·6	13·5	4·49	17,400
4	6	19	♂	Barley	29·8	62·0	32·2	2·68	90·30	2·164	14·9	17·4	2·80	10,100
		20	♀	Potato	30·2	52·8	22·6	1·88	92·56	2·189	10·3	11·6	4·10	15,800
		21	♂	Barley	30·9	70·9	40·0	3·33	119·08	2·835	14·1	16·5	2·98	10,700
		22	♂	Potato	31·4	67·6	36·2	3·02	123·32	2·911	12·4	14·0	3·41	13,400
		23	♂♂	Barley	33·7	68·7	35·0	2·92	110·89	2·648	13·2	15·5	3·17	11,400
		24	♀	Potato	33·3	68·6	35·3	2·94	109·89	2·599	13·6	15·2	3·11	12,100
		Average barley fed			—	—	35·7	3·0	—	—	14·1	16·5	2·98	10,700
		Average potato fed			—	—	31·4	2·6	—	—	12·1	13·6	3·54	13,800
		Average of all barley fed			—	—	29·5	2·5	—	—	15·6	18·7	3·56	12,900
		Average of all potato fed			—	—	23·6	2·0	—	—	12·1	14·3	4·67	18,200

(1) the average weight increase of both barley-fed and potato-fed animals was greater as the level of the casein supplement was raised, although there was little difference in food intake between the subgroups; (2) animals receiving the larger casein supplements made better use of the food energy; though the animals which received the higher levels of casein were less deficient in protein than those which received less casein, their average food intake was not significantly greater. It would therefore seem as if the food consumption of the potato-fed pigs was limited not by the lack of protein but rather

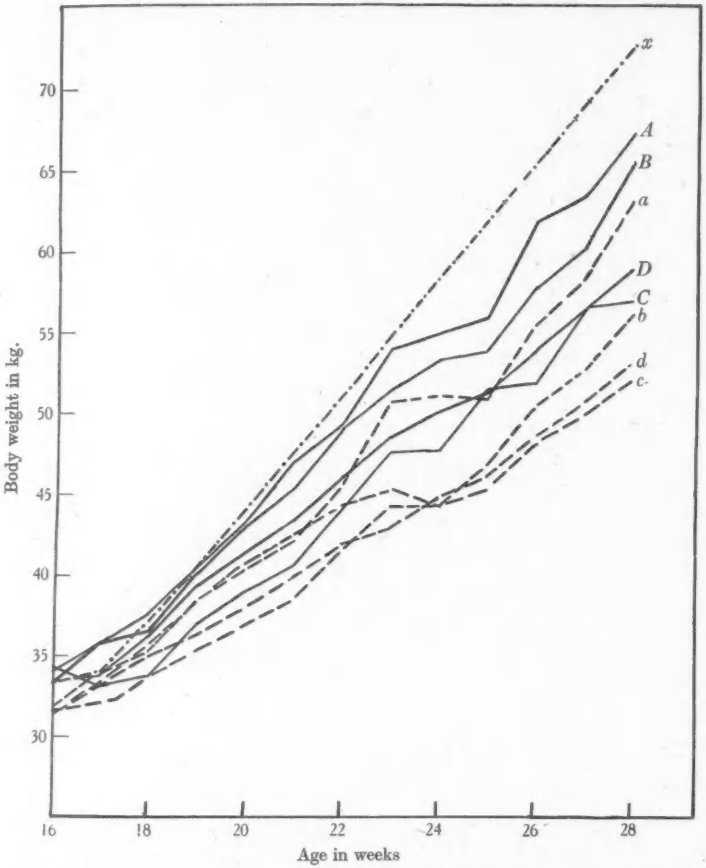

Fig. 1. Average growth curves of pigs in eight subgroups on diets containing barley and potato respectively as staple, with varying proportions of casein. —— signifies barley; ---- signifies potato. Curves *A*, *B*, *C*, *D*: pigs on barley ration + 6, 3, 1% and no casein respectively. Curves *a*, *b*, *c*, *d*: pigs on potato ration + 6, 3, 1% and no casein respectively. Curve x = average growth curve for Wessex Saddleback pigs on ordinary commercial pig feed.

(3) the differences in weight increase between barley-fed and potato-fed pigs were roughly similar at all levels of casein intake; (4) the casein therefore supplemented barley N and potato N about equally.

If the food intake of the barley-fed pigs had not been limited to that of the potato-fed pigs, the differences in weight increase between the two groups would have been considerably greater. Although the animals which received the higher levels of casein were less deficient in protein than those which received less casein, their average food intake was not significantly greater. It would therefore seem as if the food consumption of the potato-fed pigs was limited not by the lack of protein but rather by their inability to consume more of their very bulky ration. In other words, this is one of those cases to which Mitchell has frequently drawn attention, in which appetite does not run parallel with the protein efficiency of the ration; and fallacious results would have been obtained had not the food intake of the barley-fed animals been controlled. Since, on the average, the level of casein in the diet

had little effect on food intake, it was not found necessary, as had been anticipated, to equalize the food intakes of the different subgroups.

From Table 5 and Fig. 1 it appears that a supplement of 1 or 3 % of casein to the barley ration gave approximately the same rate of growth increase, and utilization of digestible energy, as a supplement of 6 % casein to the potato ration. In commercial feeding practice, therefore, it would be necessary to feed more than twice as much protein concentrate when potato is substituted for a barley ration of equal N content. Under conditions in which there might be a depression of the digestibility of N consequent on the substitution of potato for barley, it would be necessary to give still more protein concentrate. In practice the growth of the potato-fed pig would be limited by the bulkiness of the ration as well as by its deficiency of N of good biological value.

Section 2. DIGESTIBILITY EXPERIMENTS

METHODS

Six crates were made by placing the individual feeding pens upon wooden tables covered with galvanized iron. Holes were bored through the tables to drain off the urine. The pigs were prevented from moving backwards and forwards by horizontal bars fixed across the back of the pens, and adjusted to fit each pig. The animals soon learnt that the forward end of the crate, where the food appeared, was the more interesting, and there was no trouble from their turning movements in the cage during the collection periods.

After the experiments on growth were concluded, the animals continued to receive the same experimental rations until they were required for the digestibility trials. In all these trials there was a 4-day preliminary period, followed by a 10-day collection period. Food intake was adjusted to the amount which the animals were expected to finish, and was maintained at this level throughout the experiment. There were no refusals of food. The faeces were collected at intervals during the day into buckets placed behind the crates, and the animals conveniently refrained from excreting faeces during the night. The faeces were preserved by moistening with about 2 l. of *N* H_2SO_4 and analyzed for total N by the Kjeldahl method, and for gross energy in the bomb-calorimeter. Samples of the boiled potatoes were analyzed daily for dry matter and N; the gross energy was determined on a bulk dried sample.

Hogs only were utilized for these experiments, two potato-fed animals and one barley-fed animal being selected from each subgroup. It was necessary to use pig 12, a potato-fed animal, for tests with both 3 and 6 % casein diets, as two of the potato-fed pigs in the subgroup receiving 6 % of casein were gilts; pig 12 had its ration changed between the two tests.

In addition, pigs 11, 12, 17 and 18 were utilized in a further trial (see below) in which approximately half of the ration consisted of boiled potatoes and potato flakes, and approximately half of barley meal. Pigs 11 and 12 also received 6 % casein, while pigs 17 and 18 did not receive any.

RESULTS

Tables 6 and 7 give details of food intakes and faecal excretions, and (apparent) digestibility coefficients. The digestibilities of the N and energy of potato and barley are computed on the assumption that the casein, yeast extract, cod-liver oil, and starch were entirely digested. The digestibility coefficients obtained from tests with different pigs were in close agreement. Pig 8, on the potato ration with 3 % casein, scoured during the experiment, but this did not apparently influence the digestibility coefficients, which were the same as those of its fellow pig 12 on the same ration. In contradistinction to the results of Fingerling [1932], and of Woodman & Evans [1939], the presence of the casein in some of the potato rations did not depress the digestibility either of the N or of the gross energy. The digestibility of the latter was, in fact, high and independent of casein level. The coefficients averaged 93·7 % for energy, and 81·5 % for N, of the potato, and 82·3 % for energy, and 79·1 % for the N, of the barley.

When this experiment was completed, it occurred to us that, although the digestibility of the potato rations had not been lowered by feeding casein in combination with the potato, it might have been depressed if barley had been fed in combination with the potato, either with or without casein; Woodman & Evans [1939] and Fingerling [1932], in the majority of their trials, used barley meal in the basal ration. We therefore undertook a further digestibility trial on pigs 11, 12, 17 and 18. The first batch of fresh 'Majestic' potatoes had by this time deteriorated and, when cooked, usually blackened badly. These potatoes were, however, still palatable, and were given to pigs 11 and 17, while those of the second batch were fed to pigs 12 and 18.

Table 8 gives the results obtained. In computing the digestibility of the potatoes, the casein, yeast extract, cod-liver oil and starch were again assumed to be entirely digested. The mean coefficients obtained for the barley meal in the first series of experiments, viz. 82·3 % for the gross energy and 79·1 % for the N, were taken as the digestibility of the barley meal in the mixed rations. Any associative effect would thus be debited or credited to the boiled potatoes and potato flakes. The average digestibility for the potato energy in the mixed rations was calculated to be 91·0 % with no casein and 92·5 % with 6 % of casein, while that of the N averaged 75·5 and 80·7 % respectively. Thus, addition of casein to the potato ration when barley meal also was present had not depressed the digestibility

Table 6. *'Apparent' digestibility of the nitrogen and energy of potato ration*

Group	Casein in ration (%)	Pig no.	Total dry matter (kg.)	Nitrogen intake (g.) from: Casein	Yeast extract	Potatoes	Total ration	Gross energy intake (kg. cal.) from: Potatoes	Total ration	Nitrogen (g.) in: Faeces	Digested total ration	Digested potato	Gross energy (kg. cal.) in: Faeces	Digested total ration	Digested potato	Digestibility coefficients of: N in total ration	N in potato	Energy in total ration	Energy in potato
1	0	2	15·682	—	4·4	239·0	243·4	57,261	63,363	44·9	198·5	194·1	3550	59,813	53,711	81·6	81·2	94·4	93·8
	0	4	11·955	—	4·4	182·0	186·4	43,625	48,305	28·5	157·9	153·5	2494	45,811	41,141	84·7	84·3	94·8	94·3
2	1	16	9·631	14·2	4·4	147·6	166·2	35,046	39,501	35·3	187·3	161·0	2662	36,389	32,384	79·7	77·1	93·2	92·4
	1	18	14·740	21·9	4·4	196·3	222·6	53,825	59,771	39·7	241·3	174·8	2916	56,855	50,909	84·1	82·0	95·1	94·6
3	3	8	13·994	62·1	4·4	214·5	281·0	50,917	57,140	38·9	227·7	157·4	3280	53,860	47,637	85·9	81·5	94·3	93·6
	3	12	14·740	65·9	4·4	196·3	266·6	53,825	60,192	41·8	343·5	199·5	3454	56,738	50,371	85·4	80·2	94·3	93·6
4	6	12	15·749	139·6	4·4	241·3	385·3	57,330	64,976	39·7	324·5	188·3	3860	61,116	53,470	89·2	82·7	94·1	93·3
	6	22	14·874	131·8	4·4	228·0	364·2	54,126	61,366	39·7	324·5	188·3	3447	57,919	50,679	89·1	82·6	94·4	93·6
															Average	85·0	81·5	94·3	93·7

Table 7. *'Apparent' digestibility of the nitrogen and energy of barley-meal ration*

Casein in diet (%)	Pig no.	Food intake (kg. dry matter)	Nitrogen intake (g.) in: Barley meal	Casein	Yeast	Total ration	Gross energy intake: As barley (kg. cal.)	Total ration (kg. cal.)	Nitrogen (g.) in: Faeces	Digested total ration	Digested barley	Gross energy (kg. cal.) in: Faeces	Digested total ration	Digested barley	Digestibility coefficients of: N in total ration	N in barley	Energy in total ration	Energy in barley
0	3	12·374	184·9	—	4·4	189·3	41,453	51,835	35·4	153·9	149·5	6,471	45,364	34,982	81·3	80·9	87·5	84·4
1	17	15·947	238·2	24·1	4·4	266·7	53,422	67,041	49·2	217·5	189·0	9,822	57,219	43,600	81·6	79·3	85·3	81·6
3	11	15·947	238·2	72·7	4·4	315·2	53,422	67,504	48·2	267·0	190·0	9,029	58,475	44,393	84·7	79·8	86·6	83·1
6	21	15·085	225·4	137·1	4·4	366·9	50,535	64,519	53·6	313·3	171·8	10,138	54,381	40,397	85·4	76·2	84·3	79·9
															Average	83·2	79·1	85·9	82·3

Table 8. *'Apparent' digestibility of the nitrogen and energy of potatoes when fed in a mixed diet consisting of potatoes, barley meal and casein**

Group	Pig no.	Food intake (kg. dry matter): Potatoes	Barley ration	Total ration	Nitrogen intake (g.) in: Barley	Casein	Yeast	Gross energy intake (kg. cal.) in: Potatoes	Total ration	Nitrogen (g.) in: Faeces	Digested total ration	Faeces due to potatoes	Digested potato	Gross energy (kg. cal.) in: Faeces due to potatoes	Total faeces	Digestibility coefficients of: N in total ration	N in potato	Energy in total ration	Energy in potato
0% casein	17	8·338	7·974	16·312	119·1	—	4·4	123·9	247·4	30,657	26,713	67,224	56·9	190·5	31·9	92·0	3143	8141	77·0	74·3	87·9	89·8
	18	7·792	7·974	15·766	119·1	—	4·4	120·0	243·5	28,322	26,713	64,888	49·6	193·9	24·6	95·4	2246	6974	79·6	76·7	89·3	92·1
6% casein	11	8·338	7·974	16·312	119·1	142·3	4·4	123·9	389·7	30,657	26,713	68,593	51·5	338·2	26·5	97·4	2482	7210	86·7	78·6	89·6	91·9
	12	7·792	7·974	15·766	119·1	142·3	4·4	120·0	385·0	28,322	26,713	66,257	46·2	339·6	20·7	99·3	1977	6725	88·0	82·8	89·9	93·0

* The digestibility of potato is calculated by assuming both energy and N of casein and yeast to be 100% digestible, energy of barley meal to be 82·3% digestible and N of barley meal to be 79·1% digestible. The potatoes fed to pigs 11 and 17 were from batch 1 and those fed to pigs 12 and 18 from batch 2.

of either energy or N. We had not expected that there would be any effect on the digestibility of the energy, but had anticipated from the results obtained by Fingerling and by Woodman & Evans that the digestibility of the N might be lowered. The average digestibility coefficients of the potato in the rations containing the second batch of 'Majestic' potatoes (pigs 12 and 18) were 92·6 % for energy and 80·1 % for N, compared with average values of 93·7 and 81·5 % obtained with the same potatoes in the first series of trials (Table 6). The presence of the barley with the potatoes, therefore, caused no lowering of the digestibility of the ration. In short, these three feeding stuffs, potatoes, barley, and casein, gave the same digestibility coefficients when fed in a mixture as they gave, or in the case of casein, were assumed to give, when fed separately.

Our experiments, therefore, do not shed any light on the perplexingly low digestibility coefficients of potato N encountered by other workers. Both Fingerling and Woodman & Evans included fish meal in their basal rations and had we also used this concentrate in place of casein our results might possibly have been different. It is more probable, however, that differences between different samples of potatoes or methods of processing were responsible for the discrepancy. The digestibility of potatoes in the rations containing boiled potatoes from first batch of 'Majestics' averaged 90·9 % for energy and 76·9 % for N (pigs 11 and 17, Table 8); that of the 'Majestic' potatoes of the second batch averaged 93·7 % for energy and 81·5 % for nitrogen (see Table 6). There was, therefore, little difference in digestibility between the two samples of 'Majestic' potatoes which we used. Since the figures for potato digestibility given in Table 6 were obtained from experiments in which there were more animals, and in which there was no admixture with barley meal, they are considered to be the more accurate, and these figures only have been employed in the computation of the digestible nutrients in the growth experiments described above.

SUMMARY

1. The value of the nitrogen of the potato was compared with that of barley for supporting growth of 'fattening pigs'. The observations were made upon 12 pairs of similar animals, one of each pair receiving a potato, and the other a barley, diet.

2. The rations compared contained equal amounts of nitrogen derived from potato or barley, with and without supplementary nitrogen from casein. Half of the potato ration was composed of fresh boiled (Majestic) potatoes and half of commercial dried potato flakes. The total nitrogen of the diets varied from 1·5 % when no casein was given, to 2·4 % when 6 % casein was included, the figures being calculated on the dry weight.

3. The food intake, on dry weight basis, of each barley-fed pig was limited to the amount consumed by its potato-fed partner.

4. The value of the potato nitrogen, whether fed alone or supplemented with that of casein, was consistently inferior to that of the barley nitrogen. The kg. weight increase per kg. digestible nitrogen intake varied from 16·5 to 20·3 for barley rations and from 13·5 to 15·2 for corresponding potato rations. The kg. (dry) food ingested, per kg. weight increase, varied from 3·0 to 4·1 for the barley, and from 3·5 to 5·6 for the potato, rations. The conversion of food into live weight was thus more economical in the case of barley.

5. Addition of casein increased the economy in the conversion of food into live weight to about the same slight extent when either potatoes or barley were fed. The conclusion drawn is that casein nitrogen supplements equally that of potatoes and barley.

6. Some evidence was obtained that the potato-fed pigs were fatter for their carcass weight than the barley-fed pigs. Those which received no casein or only 1 % in their diet were also relatively fatter for their body weight than those receiving 3 or 6 % (see Appendix 1).

7. The average values for the coefficient of (apparent) digestibility of a mixture in equal parts of potato flakes and fresh boiled potatoes was 93·7 % for energy and 81·5 % for total nitrogen. Corresponding figures for barley were 82·3 and 79·1 %.

8. The digestibility of potatoes was unaffected by admixture with casein up to 6 % of the diet, assuming the latter to be entirely digested. It was also unaffected by admixture with barley to about 50 % of the diet, assuming that the coefficient of digestibility of the barley was the same as that ascertained when the barley was fed alone or with casein.

9. The rate of increase of weight when 3 % casein was added to the barley ration (the total nitrogen being 1·95 %) was about the same as when 6 % was added to the potato ration (the total nitrogen being 2·4 %). The intake of food was not very different in the two groups.

In conclusion we wish to thank Sir Charles Martin not only for his hospitality at Roebuck House, Chesterton, but also for constant help and advice throughout the work, Professor Dalling for the facilities given at the Institute of Animal Pathology where the pigs were kept, and Dr Harriette Chick for her help in preparation of this paper. Dr E. H. Callow, of the Low Temperature Research Station, Cambridge, gave us valuable assistance in the interpretation of the carcass data.

to be 70·1 % digestible. The potatoes fed to pigs 11 and 17 were from batch 1 and those fed to pigs 12 and 18 from batch 2.

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We are indebted to the Cambridge University Farm for the loan of the individual pens in which the pigs were fed and to Messrs Greene King for the yeast from which the yeast extract was prepared. Our thanks are also due to Mr G. A. Childs (now Sergeant in R.A.F.) for help in the feeding of the pigs, analyses of materials and calculations of results. Mr A. Tyrell was responsible for the care of the experimental animals and their quarters.

Appendix 1. Examination of Carcasses. (By J. C. D. HUTCHINSON and A. N. WORDEN)

As described in the main portion of the text, not all the pigs were killed at the termination of the growth experiments, the majority being required for the subsequent digestibility trials. During the first of the two digestibility trials, pig 12 received a diet containing 6 % casein instead of 3 % as previously, while during the second trials pigs 11, 12, 17 and 18 received mixed rations of barley and potato with or without casein. The food intake (on dry matter basis) of the pigs fed, respectively, on potato and barley rations continued to be paired, however, and the animals were killed off in pairs as soon as each digestibility trial was concluded.

The carcass measurements are recorded in Table 9. The carcass included the head, feet, flare fat and kidneys, and was weighed within 30 min.

Table 9. *Carcass measurements*

Pig no.	% casein in diet	Warm carcass weight (lb.)	Length of body cavity (mm.)	Back-fat (mm.) A	B	C_3	C_2	C_1	Calculated value of T (mm.)	$B-T$ (mm.)
1 ♂ B	0	138	822	54	27	42	39	43	21·5	5·5
2 ♂ P		129	849	51	26	31	30	37	20·2	5·8
3 ♂ B		109	800	49	20	35	29	34	17·2	2·8
4 ♂ P		96	760	43	20	32	31	33	15·3	4·7
5 ♀ B		87	646	38	13	22	15	18	14·0	(−1·0)
6 ♂ P		75	612	43	19	29	27	25	12·2	6·8
	Average: Barley-fed		756	—	20·0	—	—	—	17·6	2·4
	Potato-fed		740	—	21·6	—	—	—	15·8	5·8
13 ♂ B	1	113	713	44	18	25	21	26	17·8	0·2
14 ♀ P		99	652	45	18	24	20	23	15·8	2·2
15 ♀ B		86	760	34	15	24	21	23	13·8	1·2
16 ♂ P		81	746	45	17	28	24	31	13·1	3·9
17 ♂ B		154	874	63	29	41	41	47	23·8	4·2
18 ♂ P		147	837	54	29	40	43	39	22·7	6·3
	Average: Barley-fed		782	—	20·6	—	—	—	18·4	2·2
	Potato-fed		745	—	21·3	—	—	—	17·2	4·1
7 ♀ B	3	139	845	45	19	27	21	28	21·7	(−2·7)
8 ♂ P		112	790	42	23	34	26	39	17·5	5·5
9 ♂ B		108	732	40	16	26	19	30	17·1	(−1·1)
10 ♀ P		96	666	37	18	26	20	26	15·3	2·7
11 ♂ B		159	886	59	26	41	30	36	24·5	1·5
12 ♂ P		148	873	51	25	35	33	38	23·0	2·0
	Average: Barley-fed		821	—	20·3	—	—	—	21·1	(−0·8)
	Potato-fed		776	—	22·0	—	—	—	18·6	3·4
19 ♂ B	6	115	806	41	17	26	25	31	18·1	(−1·1)
20 ♀ P		105	791	40	20	32	27	35	16·7	3·3
21 ♂ B		146	845	44	23	33	32	39	22·6	0·4
22 ♂ P		144	852	52	21	33	27	39	22·3	(−1·3)
23 ♂ B		123	711	41	21	26	20	27	19·3	1·7
24 ♀ P		120	734	41	19	27	22	29	18·9	0·1
	Average: Barley-fed		787	—	20·3	—	—	—	20·0	0·3
	Potato-fed		792	—	20·0	—	—	—	19·3	0·7

of the death of the animal. The cold carcass weight was obtained by suitable subtraction from the warm carcass weight, i.e. 3 lb. from weights up to 143 lb., and 4 lb. from weights of 144–189 lb. The subcutaneous fat was measured at (A) the shoulder, (B) the last rib, and (C_3, C_2, C_1) the front, middle and back, respectively, of the rump muscle. The length of the body cavity was measured (on the warm carcass suspended from a gambrel) from the pubic symphysis to the junction of the sternum with the first rib [Davidson, Hammond, Swain & Wright, 1937]. The value T was calculated from the equation, $T = 1{\cdot}4 + 0{\cdot}149W$, where W is the cold carcass weight [Callow, 1942]; T represents the expected thickness of the back fat at the thinnest portion, i.e. over the last rib. The difference between the observed and calculated values ($B - T$) for this measurement are given in the last column of Table 9, and are discussed in Appendix 2.

In most cases our animals were fatter than expected, i.e. than average pigs of the same carcass weight. Callow's formula, derived from a large number of pigs of various breeds, may possibly not fit our particular Wessex Saddlebacks. Nevertheless, it is clear that the potato-fed pigs were relatively fatter, in relation to their carcass weight, than their barley-fed partners, and that the pigs which received no casein or 1% were relatively fatter than those which received 3 or 6% casein.

While it is not possible to draw definite conclusions, in view of the possible inapplicability of Callow's formula, the figures suggest that deficiency of nitrogen may affect the composition of the carcass. Those animals which received the most nitrogen of high biological value were relatively the leanest in relation to their carcass weight, thus indicating extra muscle deposition.

McMeekan [1940] could not detect any influence of the level of dietary protein on the body composition of the growing pig. The low protein diet which he used, however, supported optimal growth, and the food intakes of his animals were not limited as in our experiment. Forbes, Swift, Black & Kahlenberg [1935] and Forbes, Voris, Bratzler & Wainio [1938], working with rats whose food intake was paired, found that the nitrogen/fat ratio of the body gains increased as the dietary protein level was raised, and Hamilton [1939] arrived at the same conclusions from a similar experiment.

Appendix 2. Statistical Analysis. (By J. O. Irwin, Medical Research Council's Statistical Staff, and J. C. D. Hutchinson)

Table 10 gives the analysis of variance of (*a*) body weight increase in kg./kg. of nitrogen intake, (*b*) body weight increase in kg./kg. of digestible nitrogen intake, (*c*) dry food intake in kg., (*d*) digestible energy in kg. cal./kg. increase in body weight, and (*e*) the difference between the thickness of back fat at the last rib and the average figure for a carcass of the same weight ($B - T$ in Table 9).

In each casein subgroup every pig on a barley ration was paired with another, on a potato ration, of live weight as nearly as possible the same, and their food intakes were similarly paired. Thus the direct effect of casein level is evaluated by an analysis of variance within and between casein groups of the *sums* for two pigs. The difference between the effects of the potato and barley rations, on the other hand, and the effect of addition of casein on this difference—that is, the interaction between the main and subsidiary comparisons—were evaluated by an analysis of variance of the *differences* between the pairs of pigs. In consequence of the pairing, the error for the direct casein comparison is larger than that for the other two comparisons.

In every case there is a significant difference between the two main groups fed respectively on potatoes and barley. The direct effect of the casein additions is not significant in any of the groups but approaches significance for food intake and for digestible energy intake per kg. weight increase and for thickness of back fat. All three values are on the average less at the higher levels of casein. This fact, which agrees with the result of the previous experiment of Macrae *et al.* [1942], suggests that this non-significance is due to the relatively large error of experiment and that the effect could be shown to be real in an experiment conducted with a larger number of animals.

Table 10. *Analyses of variance*

Variation due to	Degrees of freedom	Weight increase (kg./kg. N intake) Sum of squares	Mean sq.	Variance ratio	Weight increase (kg./kg. digestible N intake) Sum of squares	Mean sq.	Variance ratio	Food intake (kg./kg. wt. increase) Sum of squares	Mean sq.	Variance ratio	Intake of digestible energy (kg. cal./kg. wt. increase) Sum of squares	Mean sq.	Variance ratio	Back fat. Difference between thickness of back fat at last rib and that calculated from warm carcass wt.† Sum of squares	Mean sq.	Variance ratio
Casein level	3	5·81	1·94	0·31	21·95	7·32	0·79	9·263	3·088	3·67	139·36	46·45	3·92	49·18	16·39	3·87
Error for casein	8	50·34	6·29	—	73·85	9·23	—	6·727	0·841	—	94·81	11·85	—	33·85	4·23	—
Potato compared with barley	1	73·50	73·50	*46·52**	119·71	119·71	*57·00*	7·404	7·404	*24·17*	164·33	64·33	*37·52*	36·75	36·75	*6·92*
Interaction	3	6·73	2·24	1·42	8·39	2·80	1·33	0·800	0·267	0·87	13·54	4·51	1·03	12·78	4·26	0·80
Error	8	12·60	1·58	—	16·83	2·10	—	2·450	0·306	—	35·02	4·38	—	42·50	5·31	—
Total	23	148·98			240·73			26·644			447·06			175·06		

* Values of the variance ratios in italics denote a significant effect.

† $(B - T)$ in Table 9.

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Respiration and Nutritional Requirements of Certain Members of the Genus *Mycobacterium*

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Much investigation [reviewed by Calmette, 1923; Long, 1923; Knight, 1936] has shown that many acid-fast bacilli can be cultivated in synthetic media. The utilization of specific carbon and nitrogen sources has also been studied [Kendall, Day & Walker, 1914; Long, 1921–22; see also Knight, 1936; Merrill, 1931].

With the exception of Uyei [1927], who employed the tubercle bacillus, authors have not defined precisely the size of the inoculum, and this is now known to be a critical factor in experiments on bacterial nutrition. Accordingly, we have reinvestigated the growth requirements of certain *Mycobacteria* in order to determine the effects of varying the size of inoculum. The use of defined inocula and other rigid conditions has made it possible to demonstrate the stimulant action of certain accessory nutrients in an unequivocal manner.

EXPERIMENTAL

Organisms. The following species were utilized: *Myco. phlei* a (Veterinary Research Station, Glenfield, N.S.W.), *Myco. phlei* b (McMaster Veterinary Laboratory, Sydney), *Myco. phlei* (no. 525), *Myco. smegmatis* (no. 523), *Myco. stercoris* (no. 3820), *Myco.* sp. *Karlinski* (no. 2071), *Myco.* sp. *leprous Kedrowsky* (no. 509), *Myco. ranae* (no. 2891), *Myco. butyricum* (no. 337), *Myco. tuberculosis hominis*, avirulent strain T_1 (Commonwealth Serum Laboratories, Melbourne; originally from the Royal Institute of Public Health, London), *Myco. tuberculosis hominis*, virulent strain H 37 (Commonwealth Serum Laboratories, Melbourne; originally from the U.S.A.). The species designated by catalogue numbers were obtained from the National Collection of Type Cultures, Lister Institute, London.

In this laboratory, stock cultures were maintained on Dorset's egg medium and subcultured every fifth week. Prior to experimental use the organisms were grown for several generations on the surface of meat infusion broth containing 5% of glycerol. Broth cultures, except those of tubercle bacilli, were 7 days old when used. All organisms were cultivated at 38°. The cultural characteristics of the three specimens of *Myco. phlei* were identical.

METHODS

Washed bacterial suspensions. In both respiratory and nutritional experiments the bacteria were removed from the surface of the broth, washed three times with sterile distilled water (by centrifugation), and a known weight resuspended in a measured volume of water. The notorious difficulty of obtaining homogeneous suspensions of acid-fast bacilli was overcome in two ways as follows:

(*a*) *Preparation of inocula for nutritional tests.* Small quantities of bacteria (up to 60 mg. wet wt.) were successfully dispersed by light hand-grinding with 0·5 ml. of water in a test-tube and plunger apparatus made from two closely fitting tubes, the contiguous surfaces of which had been carefully ground. The suspension was washed from the grinder and diluted with sterile distilled water.

(*b*) *Preparation of suspensions for manometric work.* Larger quantities (up to 2 g. wet wt.) were dispersed by gentle shaking in a small stainless steel ball-mill similar to that described by Langner & Forrester [1939]. The mill, containing bacteria, 10 ml. of sterile distilled water and three steel balls (diam. $\frac{3}{8}$ in.), was clamped to a Warburg shaker and shaken outside the thermostat for 20 min. at the rate of 40 oscillations/min. The suspension was diluted to an appropriate volume. Microscopic examination of stained smears revealed no disruption of bacterial substance or loss of acid-fast properties.

Bacterial suspensions prepared by these methods are satisfactory for the work described, and possess a degree of stability approximating to that of a yeast suspension of corresponding density. Suspensions of *Myco. smegmatis* and *Myco. stercoris* tend to flocculate more rapidly than the others.

Measurement of respiration. Oxygen consumption was measured by the manometric method of Warburg, the usual conical vessels being employed. The gas space contained oxygen and the temperature of the thermostat was 38°. The main compartment of the Warburg cup contained 1 ml. of 0·2 *M* sodium phosphate buffer, pH 7·4, to which were added 2 ml. of bacterial suspension. Substrate solutions were added from the side-bulb after 30 min. equilibration. Experiments were conducted for times ranging from 1 to 4 hr.

Oxygen uptake (Q_{O_2}) is expressed as μl. O_2 consumed/mg. dry wt. bacteria/hr. The dry weight was determined by drying 6 ml. of suspension to constant weight at 107°. 2 ml. of suspension generally contained 12–15 mg. dry weight of bacteria.

Nutritional experiments. In this work we have adhered as closely as possible to the principles stressed by Knight & Fildes [1933] and Fildes & Richardson [1935]. In adapting these principles to experiments on the slowly growing *Mycobacteria* the chief considerations were the use of a fluid synthetic medium containing substances of the highest purity, and the addition of a washed inoculum which was

large enough to produce growth in a reasonable time, but not so heavy as to carry over significant amounts of medium from the mother culture or to provide significant quantities of growth stimulants arising from cellular disintegration.

In order to ascertain the requisite inoculum and to test the ability of each organism to grow with different sources of carbon, exploratory experiments were conducted with $6 \times \frac{3}{4}$ in. test-tubes containing 5 ml. of medium. The inoculum used initially was 1 mg. wet weight of bacilli in one drop (0·05 ml.) of suspension; the result was a guide to the inoculum permissible in decisive experiments, all of which were performed in 20 ml. of medium contained in 100 ml. pyrex Erlenmeyer flasks. The depth of fluid was 6–8 mm.

A series of parallel experiments, in which two lots of bacteria were washed from 3 to 9 times, one with distilled water and the other with 0·9 % NaCl, gave identical results, showing that three washings were adequate, and that water was no more deleterious to the bacteria than saline.

In all experiments the ammonium ion was either the sole or the chief source of nitrogen. Organic materials were synthetic products, or analytical reagents where these were available; inorganic materials were of analytical grade. The following solutions were prepared:

(*a*) Basal salt mixture contained 1·0 g. of $(NH_4)_2SO_4$; 4·5 g. of KH_2PO_4; 2·0 g. of NaCl; 0·25 g. of $NaHCO_3$; 26·0 ml. of NaOH and distilled water to make the total volume 600 ml.

The pH was 7·5. The solution was distributed in test-tubes (3 ml.) or in Erlenmeyer flasks (12 ml.) and autoclaved at 15 lb. for 15 min.

(*b*) 0·2 % $MgSO_4$, $7H_2O$ (autoclaved).

(*c*) 10 % solutions of various carbon-containing substances (carbon-source) sterilized by filtration (Seitz EK) or by autoclaving, according to heat stability. Acids were used as neutral sodium salts.

(*d*) Solutions of substances under test, which were added in small volume and in low concentrations, were filtered or autoclaved according to their stability. Whenever stability was in doubt, both autoclaved and filtered specimens were tested. Acids were neutralized with NaOH, bases with HCl.

Solutions of classes (*b*), (*c*) and (*d*) were added aseptically to the distributed salt mixture, and, after all additions had been made, sterile distilled water was added to bring the final volume to 5 ml. in test-tubes, and to 20 ml. in flasks. Thus 600 ml. of original salt mixture were diluted to 1000 ml. The final concentration of magnesium sulphate was 0·04 g./litre, and that of carbon source 1–2 %.

Media were inoculated by cautious addition of 0·05 ml. of bacterial suspension to the surface, which was subsequently disturbed as little as possible. Test-tubes were slanted on trays. All vessels were plugged with cotton-wool, remaining unsealed unless experiments were prolonged beyond 7 days. Cultures were incubated at 38° and observed daily.

At the termination of an experiment the growth was transferred to tightly packed asbestos on a Gooch crucible, washed three times with distilled water, and dried to constant weight at 107°. Although this procedure involves a small loss of bacteria, the weights are sufficiently accurate for the purpose of the experiments described.

RESULTS

Respiration measurements

The oxygen consumption of several species in the presence and absence of substrates is shown in Table 1. When no substrate is added, there is an oxygen uptake which remains constant for about 2 hr. and diminishes slowly thereafter. The value of Q_{O_2} varies with the species, and bears some relationship to the rate of growth in nutrient broth. In the presence of most substrates oxygen consumption increases above the 'resting' value. There is one notable exception: citrate causes no significant change in respiration under the conditions used. Moreover, there was no oxidation of citrate when the suspension medium was phosphate saline [Krebs, 1933] or the basal salt mixture described above. Bacterial suspensions which have been heated in a boiling water-bath for 10 min. have no oxygen uptake either in presence or absence of added substrate.

Table 1. *Respiration of* Mycobacteria *with various substrates*

Substrate (0·02 *M*)	Uptake of oxygen (Q_{O_2}) with *Myco. phlei*	*Myco. smegmatis*	*Myco. stercoris*	*Myco.* sp. *Karlinski*	*Myco. ranae*	*Myco.* sp. *leprous Kedrowsky*	*Myco. butyricum*	*Myco. tuberculosis hominis* T 1
None	− 7·1	−16·2	−12·1	−18·9	−11·4	− 7·7	− 9·2	−2·0
Glucose	−27·9	−22·6	−15·3	−21·9	−32·3	− 7·9	−12·9	−3·8
Fructose	− 9·9	−26·6	−15·5	−26·3	−20·1	− 8·5	−15·5	—
Glycerol	− 8·8	−27·7	−23·0	−35·2	−20·5	−10·4	−27·1	−3·2
Sodium *dl*-lactate	−25·7	−42·6	−27·5	−36·2	−16·1	− 9·1	−11·0	−3·7
Sodium pyruvate	−11·3	−31·9	−24·2	−34·9	−35·9	− 9·8	−15·4	−2·6
Sodium acetate	− 9·3	−40·1	−25·2	−46·9	−30·6	−13·3	−22·3	−2·5
Sodium succinate	−11·2	−26·6	−22·3	−24·7	−18·1	− 8·0	− 9·3	—
Sodium citrate	− 7·1	−15·2	−11·9	−19·9	−12·6	− 7·5	− 9·1	−2·3
dl-Alanine	− 8·7	−17·4	−14·1	−22·6	−20·8	− 8·1	−10·3	—

(Medium: 0·066 *M* phosphate buffer, pH 7·4, at 38°. Gas: oxygen.)

It will be seen that the members of the acid-fast group exhibit considerable differences in their ability to oxidize the same substrate. For example, the respiration rate of *Myco. phlei* is increased three- to fourfold by glucose and lactate, whereas pyruvate, acetate, succinate and glycerol are oxidized slowly; on the other hand, the oxygen consumption of *Myco. ranae* is rapid in presence of pyruvate, acetate and glucose, but much slower in presence of lactate and succinate. *Myco. butyricum* shows preference for glycerol and acetate, whilst *Myco.* sp. *Karlinski* presents the highest rate with acetate, and distinctly lower—though still considerable—rates with glycerol, lactate and pyruvate.

Growth in synthetic media

Utilizable carbon sources

If the metabolism of the *Mycobacteria* is predominantly aerobic—as generally believed—there should be some relationship between the rate of oxidation of a given substrate and rate of growth in a synthetic medium containing that substrate as the sole source of carbon. In the experiments described in Table 2, the concentration of carbon source was 1% and the inoculum was relatively heavy (1 mg. wet wt. bacilli/tube). Gross differences in rate of growth were assessed by daily inspection. Although the first visible evidence of multiplication was often the appearance of 'bottom granules', the chief mass of growth was confined to the surface.

Selection of an inoculum suitable for critical experiments

Bacterial suspensions were prepared, by serial dilution, to contain amounts of wet bacilli varying from 10^{-1} to 10^{-8} mg./drop (0·05 ml.). Although bacilli, both clumped and isolated, could be detected microscopically, turbidity was no longer visible in a suspension of 10^{-4} mg./drop. Several tubes of glycerol broth were inoculated with each dilution, 1 drop to 5 ml. of medium. Subsequent growth indicated that suspensions containing less than 10^{-5} mg./0·05 ml. have a random distribution of bacilli, which renders them unsuitable for nutritional experiments [cf. Berg, 1941]. Suspension of 10^{-1} to 10^{-5} mg./drop gave growth in all tubes. With 10^{-1} to 10^{-3} mg./drop, growth appeared on the third day, but with 10^{-4} mg./drop not until the fifth day.

Hence an inoculum of 10^{-2} mg. was selected for most of the work, because it ensured approximately equal seedings and growth in a reasonable time. This inoculum contained about 500,000 bacilli. *Myco. ranae* is exceptional in that it shows less tendency to clump, and so it may be used in higher dilutions.

Growth with small inocula

The results of seeding 20 ml. of synthetic medium, and of glycerinated broth, with relatively small quantities of bacteria are shown in Table 3. When no growth occurred in 7 days, the plug was sealed with paraffin wax and incubation continued.

Table 2. *Growth of* Mycobacteria *in synthetic media containing various sources of carbon*

	Growth with source of carbon as 1% of						
Organism	Glucose	Fructose	Glycerol	*dl*-Lactate	Pyruvate	Acetate	Succinate
Myco. phlei	+ + +	±	±	+ + + +	+ + +	+ +	+
Myco. smegmatis	+ + +	+ + + +	+ + + +	+ + +	+ + + +	+	+
Myco. stercoris	+ +	+ + +	+ + + +	+ + + +	+ + + +	+	±
Myco. sp. *Karlinski*	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ +
Myco. ranae	+ +	+ + + +	+	±	+ + + +	+ + + +	+ +
Myco. sp. *Kedrowsky*	±	+ +	+ +	±	+ + + +	+ + +	+
Myco. butyricum	+	±	+	±	+	±	±

With citrate (0·5%) as source of carbon, no growth occurred with any organism.
Number of positive signs indicates rate of growth. ± = trace of growth.
(Nitrogen source, $(NH_4)_2SO_4$. Time, 7 days. Inoculum, 1 mg. seeded in 5 ml. of medium.)

The ability of an inoculum of 0·01 mg. to multiply to considerable proportions in 7 days is illustrated by the figures for glycerol broth. In synthetic media, however, there is no growth if the carbon source is glucose, fructose, glycerol, acetate, succinate or citrate. Lactate and pyruvate are exceptional. *Myco. phlei*, *Myco. smegmatis* and *Myco. Karlinski* grew in both lactate and pyruvate, whilst *Myco. stercoris* produced slight growth in lactate.

Table 3 also shows that an inoculum of 0·01 mg. remains viable after 7 days, and is able to attain weighable proportions with certain sources of carbon. *Myco. smegmatis* reached a growth of 7·5 mg. in succinate after 5 weeks; *Myco. Karlinski*, 15 mg.

The results demonstrate that certain species of *Mycobacteria* are able to grow when heavily seeded in synthetic media containing carbonaceous material which they can oxidize: and that, in general, the rate of growth shows some degree of correspondence to the increased oxygen uptake in phosphate buffer (Tables 1, 2). With citrate there was no increased oxygen consumption, and there was no growth in synthetic medium.

Table 3. *Growth of* Mycobacteria *in synthetic media in presence and absence of citrate*

Organism	Duration (days)	Inoculum (mg.)	Growth (mg. dry wt.) with source of carbon as 1% of: Glucose Control	Glucose Citrate	Fructose Control	Fructose Citrate	Glycerol Control	Glycerol Citrate	*dl*-Lactate Control	*dl*-Lactate Citrate	Pyruvate Control	Pyruvate Citrate	Acetate Control	Acetate Citrate	Succinate Control	Succinate Citrate	Control glycerol broth
Myco. phlei	7	0·01	Nil	10·1	Nil	Trace	Nil	Nil	15·2	24·2	15·0	20·1	Nil	9·0	Nil	Trace	46·0
		0·001	—	—	—	—	—	—	14·1	22·0	—	—	—	—	—	—	—
		0·0001	—	—	—	—	—	—	2·0	20·7	—	—	—	—	—	—	—
	21	0·01	10·2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	42	1·0	—	—	—	—	Trace	14·2	—	—	—	—	—	—	—	—	—
	56	0·01	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		1·0	—	—	—	—	8·4	—	—	—	—	—	—	—	—	—	—
Myco. smegmatis	7	0·01	Nil	14·0	Nil	23·4	Nil	17·0	42·2	44·8	13·3	27·2	Nil	9·8	Nil	16·3	50·2
	35	0·01	—	—	Nil	—	—	—	—	—	—	—	Nil	—	7·5	—	—
	63	0·01	—	—	Nil	—	—	—	—	—	—	—	Nil	—	—	—	—
Myco. stercoris	7	0·01	Nil	13·7	Nil	20·8	Nil	6·2	Trace	28·9	Nil	21·2	Nil	15·3	Nil	14·7	37·1
	21	0·01	—	—	Nil	—	Nil	—	27·8	—	Nil	—	Nil	—	Nil	—	—
	63	0·01	—	—	Nil	—	Nil	—	—	—	Nil	—	Nil	—	Nil	—	—
Myco. sp. *Karlinski*	7	0·01	Nil	8·7	Nil	9·1	Nil	6·0	3·1	18·9	3·7	16·2	Nil	2·0	Nil	4·4	33·4
		0·001	Nil	7·4	—	—	—	—	11·3	27·7	Trace	13·2	—	—	—	—	—
		0·0001	Nil	4·5	—	—	—	—	—	—	Nil	24·7	—	—	—	—	—
	42	0·01	—	—	Nil	—	Nil	—	—	—	—	—	15·0	—	Nil	—	—
	63	0·01	—	—	Nil	—	Nil	—	—	—	—	—	—	—	Nil	—	—
Myco. sp. *Kedrowsky*	7	0·01	Nil	Trace	Nil	7·0	Nil	2·5	Nil	—	3·9	5·3	Nil	3·5	Nil	—	5·8
	35	0·01	Nil	—	Nil	—	Nil	—	Nil	—	—	—	Nil	—	4·5	—	—
Myco. butyricum	7	0·01	Nil	Nil	Nil	Nil	Nil	Trace	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	22·5
	21	0·01	Nil	Nil	Nil	Nil	Nil	—	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	—
Myco. ranae	7	0·0001	Trace	5·1	Nil	Nil	Trace	0·1	Nil	10·1	1·8	9·7	Trace	2·3	Nil	Nil	6·6
	14	0·0001	—	—	Trace	8·2	—	—	—	—	—	—	—	—	Nil	Nil	—

(Nitrogen source, $(NH_4)_2SO_4$. Volume of medium, 20 ml. Conc. of sodium citrate, 0·01%.)

after 6 weeks in acetate. *Myco. stercoris*, which showed only a trace of growth in lactate after 1 week, increased to 27·8 mg. in 3 weeks, and *Myco. Kedrowsky* presented a weighable growth in pyruvate after 5 weeks, although no trace was visible at the end of the first week. *Myco. phlei* subsisting on glucose gave somewhat irregular results: in some experiments growth began after 10 days and increased to 10 mg. by the end of the third week, whereas in others no growth was obtained in 9 weeks. None of the organisms studied showed any growth in synthetic medium containing citrate as the sole source of carbon, even when the flasks were incubated for 20 weeks.

Growth usually began as small 'bottom granules' and later extended to cover the surface with a thin film, the thickness of which varied with the organism. In lactate, growth was always confined to the surface, where it began as a thin, veil-like pellicle. *Myco. phlei* presented its normal pigmentation only in the presence of glycerol [see Ingraham & Steenbock, 1935]; with other sources of carbon the pigmentation was faint. *Myco. ranae*, which is emulsified with ease, grew so rapidly at 38° that the inoculum was reduced to 0·0001 mg.

The experiments recorded in Table 3 show that the amount of growth in synthetic media is a function of both time and size of inoculum.

The stimulant effect on growth of the addition of various substances to the medium

The effect of citrate. The effect of adding a small quantity of citrate can be seen in Table 3. Uniform results, quantitatively of the same order, were obtained with five different samples of commercial citric acid, and also with a specimen of synthetic citric acid prepared by the method of Dünschmann [1891]. Controls containing citrate as the sole carbonaceous material in concentrations ranging from 0·0001 to 2·0 % have never shown the slightest trace of growth.

Table 3 shows that when the size of the inoculum and the time of the experiment are fixed arbitrarily, a small amount of citrate (0·01 %) can be a factor which determines growth in a synthetic medium; and that when some growth occurs in the absence of citrate, it always reaches greater proportions in the presence of citrate. The yields were unaffected by doubling the usual concentrations of the sources of carbon and nitrogen. The effect occurs with all organisms except tubercle bacilli (see later) and *Myco. butyricum*, which begins to grow in the presence of glycerol and citrate, but ceases after a short time.

The citrate effect was examined more extensively, *Myco. phlei* being used as a convenient organism. Table 4 illustrates the influence of concentration.

Table 4. *Growth of* Myco. phlei *in a synthetic medium containing citrate*

Incubation (days)	Inoculum (mg. wet wt.)	Conc. of citrate (%)	Bacterial growth (mg. dry wt.)
7	0·01	Nil	Nil
		0·1	7·7
		0·01	8·1
		0·001	Nil
		0·0001	Nil
30	0·01	Nil	Nil
		0·001	12·0
7	0·01	Nil	Nil
		0·01	8·1
	0·001	Nil	Nil
		0·01	6·1
	0·0001	Nil	Nil
		0·01	3·4
7	0·1	Nil	6·4
		1·0	8·6
		0·1	16·7
		0·01	13·3
		0·001	9·2

Note. In a series of 10 observations (inoculum 0·01 mg., citrate concentration 0·01 %), the mean growth in 7 days was 9·6 mg. (highest value 12·7, lowest 7·7).

(Carbon source, 1 % glucose; nitrogen source, $(NH_4)_2SO_4$; volume of medium, 20 ml.)

The optimal concentration for a short experiment was of the order 0·1–0·01 %; 1 % of citrate appears to inhibit the effect. It is noteworthy that an inoculum as small as 10^{-4} mg. can be stimulated to grow in 7 days by the presence of 0·01 % of citrate.

The experiments of Table 5 show that the citrate effect continues undiminished through serial subcultures. The organisms were inoculated as a washed suspension prepared in the usual manner. Three flasks were set up at each transfer: a control without citrate, and two containing 0·01 % of citrate. One of the resulting cultures was weighed and the other used for the next transfer.

Table 5. *Serial transfer of* Myco. phlei *in a synthetic medium*

	Growth (mg. dry wt.) with	
	Control (no citrate)	0·01 % citrate
Original culture	Nil	8·3
1st subculture	Nil	8·7
2nd ,,	Nil	8·7
3rd ,,	Nil	8·0
4th ,,	Nil	8·5
5th ,,	Nil	9·5, 9·5 (duplicates)

(Carbon source, 1 % glucose; nitrogen source, $(NH_4)_2SO_4$. Time of incubation, 7 days; inoculum, 0·01 mg. wet wt.)

The following substances related to or derived from citric acid were entirely devoid of stimulant action, when used in equivalent concentrations ($3{\cdot}9 \times 10^{-4}M$): Tricarballylic acid, aconitic acid (*trans*), trimethyl citrate, citramide, acetyl-citric acid, *dl*-citramalic acid, itaconic acid, citraconic acid, citrazinic acid, citrazinamide, and *iso*-nicotinic acid. The last three were tried because it was thought possible that ammonia might condense with citric acid to give a pyridine derivative as an intermediary metabolite.

The citric acid (initially 1·49 mg. in 20 ml.) remaining in the medium after 7 days was determined by the method of Pucher, Sherman & Vickery [1936]: about one-half of the citric acid had disappeared.

Table 6. *The stimulant effect of amino-acids on the growth of* Mycobacteria *in a synthetic medium*

Substance added (0·01%)	Growth (mg. dry wt.) with *Myco. phlei*	*Myco. smegmatis*	*Myco. stercoris*	*Myco. Kedrowsky*	*Myco. Karlinski*	*Myco. ranae*
	(*a*) Source of carbon, 1% glucose					
Syn. *dl*-Alanine	11·8	13·2	8·0	0·5	8·0	Trace
Syn. *dl*-Valine	10·4	9·1	7·7	—	6·4	Trace
Syn. *dl*-Leucine	13·6	6·6	5·4	0·7	5·6	Trace
Syn. *dl*-Norleucine	8·1	6·2	15·7	0·5	5·6	Trace
Syn. *dl*-Glutamic acid	10·6	31·6	13·9	0·8	7·3	Trace
l(−)-Tryptophan	11·0	8·5	1·6	0·2	10·3	Trace
l(−)-Proline	10·2	2·6	1·2	0·5	9·5	Trace
Syn. β-Alanine	Nil	Nil	Nil	Nil	Nil	Nil
Syn. *dl*-β-Amino-*n*-butyric acid	Nil	Nil	Nil	Nil	Nil	Nil
Syn. *dl*-δ-Amino-*n*-valeric acid	Nil	Nil	Nil	Nil	Nil	Nil
None	Nil	Nil	Nil	Nil	Nil	Nil
	(*b*) Source of carbon, 1% glycerol					
Syn. *dl*-Alanine	0·5	17·2	—	—	—	—
Syn. *dl*-Glutamic acid	1·0	13·5	6·3	—	—	—
Syn. *dl*-Leucine	0·1	—	—	—	—	—
l(−)-Proline	—	15·4	—	—	—	—
None	Nil	Nil	Nil	—	—	—

Note. (1) Controls in which the source of carbon was omitted showed no growth. (2) The prefix 'syn.' means 'synthetic'. (Main nitrogen source, $(NH_4)_2SO_4$. Time of incubation, 7 days. Inoculum, 0·01 mg. to 20 ml. of medium in all cases except *Myco. ranae*, with which the inoculum was reduced to 0·00001 mg.)

Table 7. *The stimulant effect of amino-acids on the growth of* Myco. phlei

Addition to medium		Growth (mg. dry wt.) with source of carbon as 1% of		Addition to medium		Growth (mg. dry wt.) with source of carbon as 1% of	
Substance	Conc. (%)	Glucose	Succinate	Substance	Conc. (%)	Glucose	Succinate
None	—	Nil	Nil	Syn. *dl*-Lysine	0·01	12·0	—
Syn. *dl*-Alanine	0·02	11·8	1·7	Syn. *dl*-Aspartic acid	0·01	11·6	1·9
l(+)-Alanine	0·01	11·5	—	*l*(−)-Asparagine	0·01	11·7	—
Syn. β-Alanine	0·01	Nil	Nil	Syn. *dl*-Glutamic acid	0·01	10·6	2·4
Syn. *dl*-Serine	0·01	11·2	—	*l*(+)-Glutamine	0·01	7·1	—
l(−)-Cystine	0·01	2·8	—	Syn. *dl*-α-Amino-*n*-caprylic acid	0·01	10·5	Trace
Syn. *dl*-Phenylalanine	0·02	10·3	—	*l*(+)-Arginine	0·01	7·3	—
l(−)-Phenylalanine	0·01	9·2	—	*l*(+)-Ornithine	0·01	3·5	—
d(+)-Phenylalanine	0·01	9·2	—	*l*(−)-Histidine	0·01	9·8	—
l(−)-Tyrosine	0·01	8·9	—	*l*(−)-Proline	0·01	10·2	Trace
Syn. *dl*-α-Amino-*n*-butyric acid	0·01	7·4	—	*l*(−)-Hydroxy-proline	0·01	9·1	—
Syn. *dl*-Threonine	0·01	Trace	—	Glycine	0·01	9·7	—
Syn. *dl*-Methionine	0·01	9·1	—	Glycyl-glycine	0·01	9·2	—
Syn. *dl*-Leucine	0·01	13·6	Trace	Glycyl-*l*(−)-leucine	0·01	3·6	—
Syn. *dl*-Isoleucine	0·01	7·0	—	*l*(−)-Leucyl-glycine	0·01	15·0	—
				Glutathione	0·01	8·6	—
				Casein	0·01	7·5	—
				Peptone (Witte)	0·01	10·3	—

Note. Controls in which the carbon source was omitted gave no growth.
(Main source of nitrogen, $(NH_4)_2SO_4$. Time of incubation, 7 days. Inoculum, 0·01 mg. to 20 ml. of medium.)

The effect of amino-acids, peptides and proteins. An effect similar to that of citrate is produced by small concentrations of amino-acids, dipeptides and proteins. The results are given in Tables 6 and 7.

Although amino-acids are not essential nutrients, growth is promoted by α-amino-acids, which are constituents of protein, and in which the α-amino group is free or bound in peptide linkage. The active agent appears to be either the whole amino-acid molecule, or the part of it represented by the grouping $=C(NH_2)—COOH$, since this is the only structure common to the 23 amino-acids which induced growth. There is no significant difference between the activity of those stereo-isomers that could be tested; the effect varies quantitatively, and is least with *dl*-threonine. Proline and hydroxyproline act like the α-amino-acids. Since these are protein constituents and probably convertible into α-amino-acids during metabolism, their stimulant action is not regarded as exceptional. No special importance is attached to the effect of crude proteins (peptone and casein); or to that of glutathione, since spontaneous decomposition into cysteinylglycine and pyrrolidone carboxylic acid may occur during 7 days' incubation at 38°.

When the free amino group is blocked by acetylation or benzoylation, conjugated with cholic acid, or otherwise substituted, the stimulant action is lost. β-Amino-acids, and others in which the amino group is displaced still farther from the carboxyl group, are also inactive.

Myco. phlei was tested more thoroughly than the other organisms. The following additional substances (concentration 0·01 %) failed to stimulate the growth of *Myco. phlei* subsisting on glucose: (1) sarcosine, 2:5-di-keto-piperazine, N-benzoylglycine, glycocholic acid, N-acetyl-alanine, N-benzoyl-alanine, N-phenyl-glycine, α-amino-phenylacetic acid, *dl*-ε-amino-caproic acid, *dl*-α-amino-*iso*-butyric acid, *dl*-α-amino-α-methyl-*n*-butyric acid; (2) urea, guanidine, creatine, taurine, glucosamine, and succinimide.

The effect of histamine. When a number of amines, amides and heterocyclic bases was tested, the only one that proved to be active was histamine. The magnitude of the effect is shown in Table 8.

The following were negative: (1) tyramine, tryptamine, putrescine, betaine, choline, ethanolamine, ethylamine, propylamine, *n*-butylamine, and *sec.*-octylamine; (2) acetamide, propionamide, *n*-butyramide, *n*-caproamide, and malonamide; (3) pyrrole, pyridine, pyrrolidine and piperidine. The concentration in each case was 0·01 %.

The effect of other organic substances. Many other organic substances were tested, but all were devoid of stimulant properties in so far as they produced no growth in 7 days. The organism used was *Myco. phlei*, and 0·01 mg. was seeded in 20 ml. of synthetic medium containing 1 % of glucose. Although it is unnecessary to list the whole series as negative, the fact that the following were ineffectual is recorded:

Table 8. *Stimulation of the growth of* Myco. phlei *by histamine*

Addition to medium: Substance	Conc. (%)	Bacterial growth (mg. dry wt.)
None	—	Nil
Histamine hydrochloride, synthetic (Eastman Kodak Co. Ltd.)	0·01	9·2
Histamine hydrochloride (British Drug Houses, Ltd.)	0·01	9·0
Histamine phosphate (British Drug Houses, Ltd.)	0·01	9·1
Sodium citrate	0·01	10·4

(Synthetic medium, volume 20 ml. Carbon source, 1 % glucose. Nitrogen source, $(NH_4)_2SO_4$. Inoculum, 0·01 mg. Incubation, 7 days.)

(1) Normal saturated fatty acids from C_1 to C_{18}, crotonic and oleic acids, β-hydroxy-*n*-butyric acid, *iso*-butyric and α-hydroxy-*iso*-butyric acids, normal chain dicarboxylic acids from C_2 to C_8, and hydroxy-malonic acid (concentrations, 0·01 %).

(2) Fructose monophosphate, arabinose, *d*-ribose, inositol, sorbitol, glycerol, ethylene glycol, ethanol, methanol, dihydroxy-acetone, gluconic, glyceric, lactic, pyruvic, *l*-malic, fumaric, *d*-tartaric and α-keto-glutaric acids (concentrations, 0·01 %).

(3) Uracil, adenine, adenosine, guanine, hypoxanthine, allantoin, alloxan, and hydantoin (concentrations, 0·01 %).

(4) Aneurin (100 μg.), riboflavin (50 μg.), nicotinic acid (100 μg.), phthiocol (20 μg.), pyridoxin (50 μg.), pantothenic acid (100 μg.), pyocyanine (500 μg.), cytochrome *c* (100 μg.), haemin (1 mg.), 2-methyl-1:4-naphthoquinone (saturated), ascorbic acid, indole acetic acid, β-indole propionic acid, α-naphthalene acetic acid, *p*-aminobenzoic acid and nicotinamide (all 2 mg.). The quantities in brackets are the amounts present in 20 ml. of medium.

The effect of calcium and iron. It has been shown that certain *Mycobacteria* will grow in a synthetic medium containing an oxidizable source of carbon if the seeding is sufficiently large and the incubation time sufficiently long; and that the lag period can be shortened, and growth enhanced, by addition of citrate, α-amino-acids and histamine, in low concentrations. The basis of the synthetic medium is a neutral solution containing NH_4^+, K^+, Na^+, Mg^{++}, $SO_4^=$, $PO_4^{\equiv}$, Cl^-, HCO_3^- and $CO_3^=$. Since traces of calcium and iron are present as impurities in the reagents, the question arose as to whether the amounts of these, and possibly of other elements, were optimal for growth.

Hay ash, rich in Ca^{++} and Fe^{+++}, was prepared and used according to Fildes & Richardson [1935], the result being a considerable stimulation of growth. The separate effects of Ca and Fe were

also investigated: the addition of either in low concentration was followed by growth, the character of which depended on the element. Results with *Myco. phlei* as test organism are given in Table 9. Ca was added as chloride, Fe as ferrous sulphate or ferric chloride, the solutions being added aseptically to the final medium. Ca was also added as $CaCO_3$, before autoclaving, in quantity just sufficient to saturate the basal salt mixture.

In the presence of Fe, initially Fe^{++} or Fe^{+++}, *Myco. phlei* formed a pigmented granular bottom growth, later extending to the surface as a thin yellow film; but in the presence of Ca the bottom growth developed as a slimy, whitish deposit and the surface pellicle was white. The effective concentrations are given in Table 9, which also permits a comparison with the effects of citrate and hay ash, and shows that when Mg^{++} is not added to the basal salt mixture, the stimulant effect of citrate can still be evoked. Increasing the concentration of Mg^{++} to the point of precipitating $MgNH_4PO_4$ does not stimulate growth.

Table 9. *Effect of inorganic substances on the growth of* Myco. phlei

Addition to medium: Material	Amount added to 20 ml. of medium	Bacterial growth (mg. dry wt.)
None	—	Nil
Sodium citrate	2 mg.	12·6
Hay ash	Equiv. to 1 mg. ash	7·8
Fe^{+++}	75 μg.	7·5
	50 μg.	1·8
	20 μg.	0·1
	10 μg.	Nil
Fe^{++}	50 μg.	1·5
Ca^{++}	100 μg.	7·6
	50 μg.	3·0
	20 μg.	Nil
$CaCO_3$	Saturated	7·0
Mg^{++}	50 μg.	Nil
Sodium citrate (Mg^{++} not added to basal mixture)	2 mg.	9·7

(Carbon source, 1 % glucose. Nitrogen source, $(NH_4)_2SO_4$. Inoculum, 0·01 mg. Time of incubation, 7 days.)

A control test using the ash of analytical sodium citrate gave negative results. Other ions were tested with negative results: Ni^{++}, Co^{++}, Mn^{++}, Cu^{++}, Cd^{++}, Zn^{++}, Sn^{++}, Al^{+++}, Ba^{++}, La^{+++}, Ce^{++++}, Th^{++++}, Tl^{+}, Br^{-}, I^{-}, all 50 μg./flask. Sodium silicate (2 mg.) also gave negative results.

Earlier, anomalous results were encountered in testing some possible growth stimulants, which were dissolved in the basal salt mixture and filtered through a Seitz pad. Growth occurred, although it was unexpected in view of other general findings. When the experiments were repeated by adding the unsterilized materials to autoclaved salt mixture, there was no growth. Attention was thus directed to the Seitz filtrate, which was slightly turbid and gave a test with ammonium oxalate indicating the presence of about 100 μg. of Ca^{++}/20 ml.; the Prussian blue test for Fe^{+++} [Feigl, 1937] revealed about 1–10 μg. of Fe^{+++}/20 ml., but the α-α'-dipyridyl test for Fe^{++} [Feigl, 1937] was negative. Subsequently it was discovered that both water and saline could extract detectable quantities of Ca^{++} and Fe^{+++} from filter pads in a single filtration. These findings are described elsewhere [Hunter, 1943], and it will suffice to state that they do not invalidate any observations recorded in this communication. The aseptic addition of a small volume of filtered solution (up to at least 2 ml.) to a synthetic medium, the final volume of which is 20 ml., does not convey sufficient Ca or Fe to affect the growth of *Mycobacteria* under the conditions we have employed.

Preliminary observations on the growth of tubercle bacilli and Myco. butyricum, *no.* 337

In studying the growth of tubercle bacilli (strains T_1 and H 37) in synthetic media we have found that small inocula do not propagate during an incubation period of 6 weeks. It was necessary to use either 5 mg. of washed suspension or a washed pellicle large enough to cover a 2 mm. loop. Mother cultures were 3 weeks old. Growth was examined in media containing 1 % of one of glycerol, glucose or *dl*-lactate, with and without addition of hay ash, citrate and amino-acids, separately and together. The surface of glycerol-broth medium in the control tubes was covered within 3 weeks, but growth was poor in the synthetic media, amounting to no more than a thickening of the inoculum or a slight patchy extension. Growth could be stimulated to normal dimensions by addition of small quantities of Marmite or muscle *Kochsaft*.

It has been seen that a small inoculum of *Myco. butyricum* grows poorly in the synthetic medium. Larger inocula in the form of washed surface pellicles also failed to exhibit extension in a glycerol medium to which hay ash, citrate, and amino-acid mixtures had been added. Again muscle *Kochsaft* and Marmite stimulated rapid growth approaching that of broth controls.

These observations suggest that the tubercle bacillus and *Myco. butyricum* (no. 337) have a more exacting nutrition than the other organisms studied.

DISCUSSION

When certain species of *Mycobacteria* are lightly seeded in a synthetic medium under the conditions described in this paper, it is possible to demonstrate

the effectiveness of growth stimulants which operate in concentrations of the order $1 \times 10^{-3} M$ or lower. The active substances belong to four apparently independent classes: (1) citric acid, (2) α-amino-acids, (3) histamine, (4) ions of calcium and iron. They are termed 'growth stimulants' because they do not conform to the definition of essential growth factors [Fildes & Richardson, 1935].

Many investigators have found that citric acid is an advantageous constituent of media specially designed for cultivation of tubercle bacilli. Under the conditions of our experiments low concentrations of citric acid do not stimulate the growth of tubercle bacilli, but this substance effectively reduces the lag period and promotes the growth of acid-fast saprophytes. Experiments with *Myco. phlei* show that citric acid is not oxidized as a source of energy; nor does it catalyze the oxidation of carbohydrate after the manner of the citric acid cycle [Krebs & Johnson, 1937], since intermediates of the cycle—such as α-keto-glutaric, succinic, fumaric and *l*-malic acids—do not stimulate growth.

Although citric acid is not oxidized, it disappears from the medium. It is possible that the organisms normally synthesize small amounts of citric acid from a variety of sources of carbon during the lag period. If this were so, the stimulant effect of citric acid would be explained.

The stimulant properties of amino-acids have been observed with other bacterial families, e.g. *Esch. coli* [Sahyun, Beard, Schultz, Snow & Cross, 1936] and propionic acid bacteria [Wood, Anderson & Werkman, 1938]. The possibility that citric acid is concerned in the synthesis of amino-acids was considered, but no evidence to support the suggestion was obtained.

Sauton [1912] and Long & Seibert [1926] found that Fe enhances the growth of tubercle bacilli. Our experiments show that the growth of acid-fast saprophytes can be stimulated by very low concentrations of Ca and Fe; but in the absence of added Ca^{++}, Mg^{++} or Fe^{+++} growth still occurs if citrate be present, showing that if any, or all, of these ions be essential, sufficient is provided by the impurities of analytical reagents.

It must be emphasized that the *Mycobacteria* used in this work have been cultivated on laboratory media for years and have had ample opportunity to adapt themselves to artificial conditions. Although the stock cultures have been kept on a rich medium, it is possible that freshly isolated organisms would have more exacting nutritional demands.

SUMMARY

1. The respiration of certain species of *Mycobacteria* has been determined in presence and absence of substrates. Species vary widely with respect to the respiration of 'resting' cells and to oxygen consumption in the presence of different substrates.

2. Growth of small inocula in a synthetic medium has been studied. It was found that under defined conditions the growth of some species is stimulated by citric acid, α-amino-acids, histamine, calcium and iron.

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The Antibacterial Action of some Stilbene Derivatives

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Early types of antibacterial substances were not specifically antibacterial, but were usually toxic to all living cells, and were of value only in so far as they could be employed without serious damage to the host. The search for more suitable antibacterial agents resulted in the preparation and testing of many substances of widely different constitution, and it was from the empirical yet systematic investigations of one such class of chrysoidine dyestuff that knowledge of the activity of sulphanilamide arose. The growing evidence that the antibacterial action of sulphanilyl drugs was due to competition with an enzymic metabolic process suggested to Fildes [1940] a new approach to the problem. This was the design of antibacterial agents by the synthesis of compounds with a close spatial resemblance to substances vital to, or closely associated with, bacterial growth and reproduction. These growth substances include the water-soluble vitamins of the animal world, which now appear to be essential to the life of all growing cells. The method has been recently used in a sufficiently diverse series of independent investigations to confirm the principle [McIlwain, 1943].

Other compounds which exert a pronounced physiological activity in minute concentration are the animal hormones related to cyclopentanophenanthrene; for example, oestradiol, when injected into an ovariectomized rat of 50 g. body weight in a dose of 0·2 μg., is capable of doubling the weight of the uterus; with a blood volume of 2 ml., this represents an activity at a concentration of 1 in 10^7. These compounds are not known to be hormones for the vegetable kingdom, but they are widely distributed in nature and are probably constituents of all growing cells, being found in all plants and bacteria [Aschheim & Hohlweg, 1933], as well as in all animals. It seems to us that these compounds can readily be incorporated into the living cell, and that such molecules constitute suitable material which, while not necessarily competitive with known essential systems, is capable of modification to contain known antibacterial groupings. Synthetic work in the cyclopentanophenanthrene series is difficult, and it is fortunate that the work of Dodds, Golberg, Lawson & Robinson [1939] provided, in the diethylstilboestrols, simple substances with space configurations similar to those of the naturally occurring compounds, and with their intense physiological activity.

We have prepared and tested a number of compounds in this series for antibacterial properties and have found some of them very powerfully bactericidal. As with the oestrogenic properties of compounds in this series, the antibacterial activities vary considerably with the structure of the molecule, though these variations are not altogether analogous. The compounds tested are set out in Table 1 and fall into seven main classes:

I. Deoxystilboestrol and its dihydro-derivative (1 and 2).

II. α:α′-Diethylstilbene, intermediates in its preparation and some derivatives (3–7).

III. Deoxydiethylstilboestrol, intermediates and derivatives (8–14).

IV. Diethylstilboestrol and derivatives (15–20).

V. Amino-, cyano-, amidino- and nitro-derivatives (21–29).

VI. Naturally occurring steroids (31 and 32).

VII. Penicillic acid and citrinin (33 and 34).

I–VI are poorly soluble in water and in broth; therefore saturated solutions in broth were first prepared by making use of the solubility of the compounds in 90 % (v/v) ethanol, in which a 10 % solution can be prepared. Small volumes of this solution, containing a known amount of drug, were added to measured volumes of nutrient broth and the mixture autoclaved. When a volume of 0·1 ml. was added to 100 ml. of broth, the material remained in solution, or in a very finely divided suspension, at 37°, and from the starting solution or suspension a series of concentrations of drug was made by pipetting 5 ml. into a suitable quantity of sterile broth. This series of drug broths was then inoculated with a suspension of the strains of *Staphylococcus* or *Streptococcus* used in these experiments. The inocula were adjusted to give a final count of 1000–2000 organisms/ml. of drug broth and the actual numbers present were determined by a plate count in each case.

Figures are given in Table 1 for the minimal concentration of drug which just prevents visible growth of the organism after 48 hr. incubation at 37°. Subcultures from tubes containing the minimal inhibitory concentration showed that the action of

Table 1. *Bactericidal activity of the compounds tested against strains of* Staphylococcus *and* Streptococcus

			Staphylococcus	*Streptococcus*
1.	4-OH.C_6H_4.CH=CH.C_6H_5	Hewitt *et al.* [1912]	1: 50,000	1: 50,000
2.	4-OH.C_6H_4.CH_2.CH_2.C_6H_5	Stoermer & Kippe [1903]*	1: 40,000	1: 20,000
3.	C_6H_5.CO.CHEt.C_6H_5	Janssen [1889]	1:200,000	1: 200,000
4.	C_6H_5.C(OH)Et.CHEt.C_6H_5		1: 30,000	1: 30,000
5.	C_6H_5.CEt=CEt.C_6H_5	Kharasch & Kleiman [1943]*	1: 10,000	1: 60,000
6.	C_6H_5.CHEt.CHEt.C_6H_5	Kharasch & Kleiman [1943]*	Inactive	Inactive
7.	C_6H_5.C(OH)Et.C(OH)Et.C_6H_5	Ciamician & Silber [1914]	Slight bacteriostatic activity	Inactive
8.	4-MeO.C_6H_4.CO.CHEt.C_6H_5	Dodds *et al.* [1939]	Inactive	Inactive
9.	4-MeO.C_6H_4.C(OH)Et.CHEt.C_6H_5	Dodds *et al.* [1939]	Inactive	Inactive
10.	4-MeO.C_6H_4.CHEt.CHEt.C_6H_5		Inactive	Inactive
11.	4-OH.C_6H_4.CEt=CEt.C_6H_5	Dodds *et al.* [1939]*	1:640,000	1:2,000,000
12.	4-OH.C_6H_4.CHEt.CHEt.C_6H_5		1:200,000	1: 800,000
13.	4-BzO.C_6H_4.CHEt.CHEt.C_6H_5		Inactive	Inactive
14.	4-HSO_3O.C_6H_4.CHEt.CHEt.C_6H_5		1: 3,000	1: 640,000
15.	4-OH.C_6H_4.CEt=CEt.C_6H_4.OMe-4′		1: 10,000	1: 20,000
16.	4-OH.C_6H_4.CEt=CEt.C_6H_4.OH-4′ (h.m.†)	Dodds *et al.* [1939]	1:100,000	1: 400,000
17.	4-OH.C_6H_4.CEt=CEt.C_6H_4.OH-4′ (l.m.‡)	Dodds *et al.* [1939]	1:100,000	1: 400,000
18.	4-OH.C_6H_4.CHEt.CHEt.C_6H_4.OH-4′ (h.m.)	Dodds *et al.* [1939]	1:100,000	1: 300,000
19.	4-OH.C_6H_4.CHEt.CHEt.C_6H_4.OH-4′ (l.m.)	Dodds *et al.* [1939]	1: 80,000	1: 400,000
20.	4-AcO.C_6H_4.CHEt.CHEt.C_6H_4.OAc-4′ (l.m.)	Dodds *et al.* [1939]	1: 50,000	1: 100,000
21.	C_6H_5.CO.CHEt.C_6H_4.NH_2-4		1: 8,000	1: 8,000
22.	C_6H_5.C(OH)Et.CHEt.C_6H_4.NH_2-4		1: 5,000	1: 10,000
23.	C_6H_5.CEt=CEt.C_6H_4.NH_2-4		1: 32,000	1: 128,000
24.	C_6H_5.CEt=CEt.C_6H_4.$NHSO_3$.C_6H_4.NH_2-4		1: 40,000	1: 40,000
25.	4-MeO.C_6H_4.CH=CH.C_6H_4.NO_2-4′	Hewitt *et al.* [1912]	1: 10,000	1: 10,000
26.	4-OH.C_6H_4.CH=CH.C_6H_4.NO_2-4′	Cullinane [1923]	1: 20,000	1: 20,000
27.	4-OH.C_6H_4.CH=CH.C_6H_4.NH_2-4′		Inactive	Slight activity
28.	4-OH.C_6H_4.CH=CH.C_6H_4.CN-4′		Inactive	Slight activity
29.	4-OH.C_6H_4.CH=CH.C_6H_4.C(NH)(NH_2)-4′		1: 30,000	1: 30,000
30.	4-OH.C_6H_4.CO.CHOH.CH_3	Auwers & Noll [1938]	1: 600	1: 600
31.	Oestrone		1: 33,000	1: 33,000
32.	Oestradiol		1: 15,000	1: 40,000
33.	Penicillic acid	Oxford [1942]	1: 1,500	—
34.	Citrinin	Oxford [1942]	1: 15,000	—

* Revised method of preparation given. † h.m. = high-melting. ‡ l.m. = low-melting.

the drugs was usually bactericidal, but bacteriostasis was also shown in some cases at lower concentrations by the appearance of delayed growth 24–48 hr. after inoculation.

When a compound is stated in Table 1 to be inactive, it indicates that a saturated solution obtained by filtering broth suspension containing the drug in a concentration of 1/100,000 failed to prevent the growth of a small number of organisms.

The antibacterial activities of deoxydiethylstilboestrol and its dihydro-derivative were tested against a number of gram-negative organisms, but both compounds were generally inactive in the highest concentrations obtainable.

So that our results may be comparative we have included in the series estimates of penicillic acid and citrinin; with our strain of *Staphylococcus* penicillic acid is bactericidal at a concentration of 1:1500 and citrinin at 1:15,000, so that our most active specimen, 4-OH.C_6H_4.CEt=CEt.C_6H_5 (11), is some 40 times as active as the latter.

Deoxydiethylstilboestrol is soluble in soap solutions and its germicidal action may be compared with soap solutions of the chlorinated phenols. The results obtained in a Rideal-Walker type test, where in addition to *Eberthella typhosa*, strains of *Streptococcus*, *Staphylococcus*, and *Pseudomonas aeruginosa* (*Ps. pyocyanea*) were examined, are given in Table 2. The minimal concentration required to kill gives the concentration of disinfectant in which life

Table 2. *Comparative bactericidal properties of phenol, chlorinated phenols and deoxydiethylstilboestrol*

	Minimal concentration required to kill			
Material*	*Streptococcus pyogenes* (P 90)	*Staphylococcus aureus* (133)	*Ps. aeruginosa* (1)	*Eberthella typhosa* (Rawlings)
1% deoxydiethylstilboestrol	1:1600	1: 5	1:10	1: 2·2
1% *p*-chloro-*m*-xylenol	1:1600	1: 5	1:10	1:27
1% *p*-chloro-*m*-cresol	1: 800	1: 2·5	1:10	1:20
5% phenol	1: 120	1:80	1:70	1:90

* The compounds were dissolved in 5% sodium oleate solution.

survives for 2½ and 5 min. but not thereafter. Deoxydiethylstilboestrol is as effective a disinfectant as chloroxylenol against gram-positive organisms but is less effective against gram-negative types.

Some of the compounds were tested in mice infected with virulent strains of staphylococci and streptococci, the drugs being administered by mouth in three doses, immediately, 5 hr., and 24 hr. after infection. The dose was 1–10 mg./g. of mouse, and no protection was afforded by any of the compounds tried, with the possible exception of

$4\text{-}HSO_3O.C_6H_4.CHEt.CHEt.C_6H_5$ (14)

which showed very slight activity in this respect.

DISCUSSION

The comparison of the bactericidal activities presented in the table shows that the two most active compounds are deoxydiethylstilboestrol (11) and its dihydro-derivative (12). The factors which appear to effect the activity of the compounds tested are: (*a*) the carbon skeleton; (*b*) the substituent groups; (*c*) the degree of unsaturation of the central bridge.

The preferred spatial configuration of the diethylstilbene type as compared with the unalkylated stilbene type is shown by a comparison of

$4\text{-}OH.C_6H_4.CH=CH.C_6H_5$ (1)—1:50,000,

and

$4\text{-}OH.C_6H_4.CEt=CEt.C_6H_5$ (11)—1:640,000;
$4\text{-}OH.C_6H_4.CH_2.CH_2.C_6H_5$ (2)—1:40,000,

and

$4\text{-}OH.C_6H_4.CHEt.CHEt.C_6H_5$ (12)—1:200,000.

(The ratios here and in subsequent comparisons refer to the minimal bactericidal dilutions (Table 1) of these compounds against *Staphylococcus*.)

The effect of the hydroxy group in the 4-position is shown by the pairs:

$C_6H_5.CEt=CEt.C_6H_5$ (5)—1:10,000,

and

$4\text{-}OH.C_6H_4.CEt=CEt.C_6H_5$ (11)—1:640,000;
$C_6H_5.CHEt.CHEt.C_6H_5$ (6)—inactive,

and

$4\text{-}OH.C_6H_4.CHEt.CHEt.C_6H_5$ (12)—1:200,000.

This evidence is of interest in that bactericidal activity and oestrogenic activity both require the presence of the 4-hydroxy group.

However, analogy with oestrogenic activity ends here, since the reduction of the centre double-bond diminishes, not enhances, the antibacterial activity, e.g.

$4\text{-}OH.C_6H_4.CH=CH.C_6H_5$ (1)—1:50,000,

and

$4\text{-}OH.C_6H_4.CH_2.CH_2.C_6H_5$ (2)—1:40,000;
$C_6H_5.CEt=CEt.C_6H_5$ (5)—1:10,000,

and

$C_6H_5.CHEt.CHEt.C_6H_5$ (6)—inactive;
$4\text{-}OH.C_6H_4.CEt=CEt.C_6H_5$ (11)—1:640,000,

and

$4\text{-}OH.C_6H_4.CHEt.CHEt.C_6H_5$ (12)—1:200,000;

and the introduction of a hydroxyl into the second ring diminishes the activity:

$4\text{-}OH.C_6H_4.CEt=CEt.C_6H_5$ (11)—1:640,000,

and

$4\text{-}OH.C_6H_4.CEt=CEt.C_6H_4.OH\text{-}4'$ (16 and 17)—1:100,000;
$4\text{-}OH.C_6H_4.CHEt.CHEt.C_6H_5$ (12)—1:200,000,

and

$4\text{-}OH.C_6H_4.CHEt.CHEt.C_6H_4.OH\text{-}4'$ (18 and 19)—1:100,000 and 1:80,000.

The two forms of diethylstilboestrol (16 and 17) have similar antibacterial activities, whereas the normal form is about 14 times as potent an oestrogen as *ψ*-stilboestrol. Thus while both molecules may be readily accepted by cells, both may prove bactericidal while only one is capable of fulfilling the oestrogenic function to a marked degree. (This is interesting if indirect evidence that the lack of oestrogenic activity of *ψ*-stilboestrol is not due to any 'hindrance' effect of the cell membrane.) Furthermore, high-melting hexoestrol (18) is very much more active oestrogenically than its lower melting stereoisomer (19), but their antibacterial activities are very close (1:100,000 and 1:80,000 respectively).

Conversion of the 4-hydroxyl to methoxyl destroys the activity completely, as shown by the inactivity of $4\text{-}MeO.C_6H_4.CHEt.CHEt.C_6H_5$ (10); if there are two substituent hydroxyls, conversion of one of them to methoxyl greatly reduces the activity:

$4\text{-}OH.C_6H_4.CEt=CEt.C_6H_4.OH\text{-}4'$ (16)—1:100,000,

and

$4\text{-}OH.C_6H_4.CEt=CEt.C_6H_4.OMe\text{-}4'$ (15)—1:10,000;

cf. also

$4\text{-}OH.C_6H_4.CH=CH.C_6H_4.NO_2\text{-}4'$ (26)—1:20,000,

and

$4\text{-}MeO.C_6H_4.CH=CH.C_6H_4.NO_2\text{-}4'$ (25)—1:10,000.

The influence of hydroxy groups in the centre of the molecule is interesting.

$$C_6H_5.CO.CHEt.C_6H_5 \ (3)$$

which, in its enolic form, is

$$C_6H_5.C(OH)=CEt.C_6H_5,$$

is bactericidal at 1:200,000;

$$C_6H_5.C(OH)Et.CHEt.C_6H_5 \ (4),$$

which contains a hydroxyl but no double bond, at 1:30,000; the analogous hydrocarbons

$$C_6H_5.CEt=CEt.C_6H_5 \ (5)$$

and $$C_6H_5.CHEt.CHEt.C_6H_5 \ (6)$$

are bactericidal at 1:10,000, and inactive, respectively. The provision of a second hydroxyl in the centre, cf.

$$C_6H_5.C(OH)Et.C(OH)Et.C_6H_5 \ (7),$$

lowers the activity.

In the 4-amino series the effect is not quite the same. Here compounds containing the hydroxyl in the centre are less active than the parent substance, though the activity is still greater in presence of a double bond. Thus

$$C_6H_5.CO.CHEt.C_6H_4.NH_2\text{-}4 \ (21)$$

is active at 1:8000 and

$$C_6H_5.C(OH)Et.CHEt.C_6H_4.NH_2\text{-}4 \ (22)$$

at 1:5000. However, the compound

$$C_6H_5.CEt=CEt.C_6H_4.NH_2\text{-}4 \ (23)$$

is active at 1:32,000, an increase possibly to be ascribed to the amino group here freed from any interference by the hydroxyl.

Since in the diethylstilboestrol series the maximal oestrogenic activity appears to be associated with compounds containing two hydroxyls in the 4:4′-positions, it seemed possible that some group present in the 4′-position might enhance the activity of 4-hydroxystilbene. Accordingly, a series of 4-hydroxystilbenes were prepared with the groups

$$—CN, —NH_2, —C\begin{matrix}=NH\\ \diagdown NH_2\end{matrix} \text{ and } —NO_2$$

(27, 28, 29 and 26 respectively) in the 4′-position. However, the last two depressed the activity slightly, whilst the first two practically destroyed it.

CHEMICAL METHODS

4-Hydroxydibenzyl (2). This compound was prepared by a more convenient method than that given in the literature [Stoermer & Kippe, 1903; Freund & Remse, 1890; Späth, 1913]. 4-Methoxydeoxybenzoin (7 g.) was refluxed with amalgamated zinc (30 g.), conc. HCl (100 ml.) and water (100 ml.) for 5 hr., fresh HCl being added each hour. The hot liquid was decanted from the zinc, cooled, the solid product collected, washed with water and crystallized from ethanol. 4-Methoxydibenzyl was obtained in colourless plates, m.p. 61–62°, as in the literature. For hydrolysis this ether (6·3 g.) was dissolved in an ethereal solution of methyl magnesium iodide (prepared from 0·8 g. of magnesium, and 4·0 ml. of methyl iodide in 10 ml. of ether). The mixture was evaporated to dryness and heated in an oil-bath until frothing started (*c.* 180°); as this decreased the temperature was gradually raised to 200°. After 3 hr. the mixture was decomposed with ice and HCl and the colourless insoluble product filtered, washed and crystallized (as the sodium salt) from hot aqueous 2*N* NaOH. The regenerated 4-hydroxydibenzyl crystallized from carbon tetrachloride in colourless plates (4 g.), m.p. 100–101° as in the literature.

α:β-Diethyl-α-hydroxydibenzyl (4). α-Ethyldeoxybenzoin (14·0 g.) was dissolved in ether (50 ml.) and gradually treated with a solution of ethyl magnesium bromide (from 2 g. of magnesium and 7 ml. of ethyl bromide in 30 ml. ether). The addition completed, the mixture was heated on a water-bath for 10 min., cooled, and the product decomposed with ice and HCl. The ethereal layer was separated, dried, the solvent removed and the residue distilled *in vacuo*, b.p. 185–195° at 18 mm. (13 g.). On redistillation, α:β-diethyl-α-hydroxydibenzyl was obtained as a colourless viscous oil, b.p. 182–186° at 14 mm. (Found: C, 85·0; H, 8·6%. $C_{18}H_{22}O$ requires C, 85·0; H, 8·7%.)

α:β-Diethylstilbene (5). α:β-Diethyl-α-hydroxydibenzyl was dehydrated with phosphorus trichloride [Dodds *et al.* 1939] giving α:β-diethylstilbene as a colourless mobile oil, b.p. 170° at 15 mm. (Found: C, 91·2; H, 8·4%. Calc. for $C_{18}H_{20}$, C, 91·5; H, 8·5%.) This is in agreement with Ramart-Lucas & Anagostopoulos [1928] and also with Kharasch & Kleiman [1943], but not with Rising & Zee [1928] or Carlisle & Crowfoot [1941], who describe it as a crystalline solid.

α:β-Diethyldibenzyl (6). Diethylstilbene (5·6 g.) was shaken in an atmosphere of hydrogen with platinum oxide (0·5 g.) and acetone (50 ml.). The solution was filtered, evaporated, and the residue (4·5 g.; m.p. 74–76°) recrystallized from methanol. α:β-Diethyldibenzyl was obtained in long needles, m.p. 83–84°. (Found: C, 90·7; H, 9·1%. Calc. for $C_{18}H_{22}$, C, 90·7; H, 9·3%.) This melting-point is much lower than values recorded in the literature. Moritz & Wolfenstein [1899] give 88°; Späth [1913] gives 92–93°.

γ:δ-Dihydroxy-γ:δ-diphenylhexane (7). Propiophenone (6 g.) in wet ether (100 ml.) was treated with aluminium amalgam (prepared from 6 g. aluminium powder and 9 g. mercuric chloride in 300 ml. ethanol) and, when the reaction had subsided, the mixture was refluxed on a water-bath for 45 min. The ether was evaporated and the residue crystallized repeatedly from light petroleum (b.p. 40–60°) yielding a white solid, m.p. 135–136° (Ciamician & Silber [1914] give 138–139°).

4-Methoxy-α:β-diethyldibenzyl (10). 4-Methoxy-α:β-diethylstilbene (20 g.) was shaken in acetone (100 ml.) with palladium charcoal catalyst (prepared by reducing 0·2 g. palladium chloride in presence of 3·8 g. charcoal) in hydrogen at 1 atm. pressure. After filtration the solution was evaporated and the product crystallized from ethanol; it had m.p. 89–90° (19 g.). (Found: C, 84·6; H, 8·8%. $C_{19}H_{24}O$ requires C, 85·0; H, 9·0%.)

4-Methoxy-α:β-diethylstilbene. δ-Phenyl-γ-anisylhexan-γ-ol, without purification, distilled at ordinary pressure with elimination of water, at 325–330°. The product, crystallized from ethanol, had m.p. 79–80°. (Found: C, 85·5; H, 8·2%. $C_{19}H_{22}O$ requires C, 85·7; H, 8·3%); the yield was quantitative.

4-Hydroxy-α:β-diethylstilbene (11). To a solution of methylmagnesium iodide (prepared from 5 g. of magnesium and

12 ml. of methyl iodide in 200 ml. of dry ether) was added 4-methoxydiethylstilbene (20 g.), and after evaporation of the ether the mixture was heated in an oil-bath, first at 170° until frothing subsided, and then at 200° for 30 min. After decomposition with water and HCl, the product was extracted with carbon tetrachloride and the solution concentrated to crystallize; m.p. 125–127°. (Found: C, 84·9; H, 8·0%. Calc. for $C_{18}H_{20}O$, C, 85·7; H, 8·0%.)

4-Hydroxy-α:β-diethyldibenzyl (12). To a solution of methylmagnesium iodide (prepared from 7·5 g. of magnesium and 18 ml. of methyl iodide in 300 ml. of ether) was added 4-methoxydiethyldibenzyl (30 g.) and, after evaporation of the ether, the mixture was heated in an oil-bath, first to 160° and then, when the reaction had subsided, to 195° for 30 min. After decomposition with water and acidification with HCl, the precipitate was crystallized from carbon tetrachloride; m.p. 139–140° (15 g.). (Found: C, 85·1; H, 8·7%. $C_{18}H_{22}O$ requires C, 85·0; H, 8·7%.) The *benzoate* (13), prepared by the Schotten-Baumann method or by the action of benzoyl chloride on a pyridine solution of the phenol, crystallized from methanol in long prisms, m.p. 110°. (Found: C, 83·8; H, 7·1%. $C_{25}H_{26}O$ requires C, 83·8; H, 7·3%.)

The *sulphonate* (14) was obtained as its pyridine salt by the action of chlorosulphonic acid (1·5 ml.) on a pyridine solution of the parent phenol (1·0 g.). As it was somewhat unstable to heat, it was purified by dissolution in cold chloroform and precipitation with ligroin. Colourless plates, m.p. 195–196°. (Found: C, 66·3; H, 6·4; S, 7·8; N, 3·5%. $C_{23}H_{27}O_4NS$ requires C, 66·8; H, 6·5; S, 7·7; N, 3·4%.)

Diethylstilboestrol monomethyl ether (15) was obtained as a by-product in the production of diethylstilboestrol by hydrolysis of the dimethyl ether. It crystallized from ethanol; m.p. 101–102°. (Found: C, 80·4; H, 7·7%. $C_{19}H_{22}O_2$ requires C, 80·8; H, 7·8%.)

4-Nitro-α-ethyldeoxybenzoin. 4-Nitrodeoxybenzoin [Petrenko-Kritschenko, 1892; Womack, Campbell & Dodds, 1938] (35 g.), was added to a hot solution of sodium (3·9 g.) in absolute ethanol (800 ml.), ethyl iodide (39 ml.) was added as quickly as possible to the resulting solution, and the whole refluxed until the solution was neutral (2–3 hr.). During this time the colour changed from deep purple to yellow. After pouring into water the solution was extracted with benzene, the benzene layer washed twice with water, dried over calcium chloride, and concentrated to *c.* 100 ml. This solution was treated with an equal volume of light petroleum (b.p. 60–80°), when a crystalline solid separated (31 g., m.p. 75–78°). Recrystallized from ethanol (charcoal), *4-nitro-α-ethyldeoxybenzoin* formed almost colourless prisms, m.p. 78–80°. (Found: C, 71·2; H, 5·9; N, 5·4%. $C_{16}H_{15}O_3N$ requires C, 71·4; H, 5·6; N, 5·2%.)

4-Amino-α-ethyldeoxybenzoin (21). 4-Nitro-α-ethyldeoxybenzoin (8 g.), iron filings (24 g.) and water (8 ml.) were ground into a paste, transferred to a flask together with xylene (8 ml.) and 1% $FeCl_3$ solution (12 ml.) and heated on a vigorously boiling water-bath for 3 hr., with continuous stirring. After 1½ hr., more xylene (4 ml.) was added. The mixture was allowed to cool somewhat, treated with benzene (100 ml.) and the iron oxide filtered off and washed thoroughly with a further quantity of warm benzene (100 ml.). The combined benzene layers were separated from the aqueous portion and exhaustively extracted with *N* HCl. By this means coloured resinous impurities remained in the benzene solution whilst, on making alkaline, the HCl deposited an oil which rapidly set to an almost colourless solid. This was collected, dried (6·6 g.) and crystallized (charcoal) from cyclohexane, from which *4-amino-α-ethyldeoxybenzoin* separated in sprays of needles, m.p. 128–129°. (Found: C, 80·5; H, 7·2; N, 6·0%. $C_{16}H_{17}ON$ requires C, 80·3; H, 7·1; N, 5·9%.)

4-Amino-α:β-diethyl-β-hydroxydibenzyl (22). A solution of 4-amino-α-ethyldeoxybenzoin (5·0 g.) in benzene (80 ml.) was gradually run into an ice-cooled solution of ethylmagnesium bromide (from 2 g. magnesium, 30 ml. ether and 7 ml. ethyl bromide, the ether being subsequently removed) in benzene (30 ml.). A thick yellow precipitate formed immediately. After standing overnight at room temperature the mixture was refluxed on a water-bath for 5 min., cooled and the magnesium complex decomposed with ice and ammonium chloride. A small amount of insoluble material was filtered off and the benzene layer separated, washed once with water and then extracted with HCl. The acid solution was made alkaline with ammonia, the resulting oil taken up in ether and the ethereal solution dried and evaporated. The crude residue crystallized on cooling and was purified by repeated recrystallization from a mixture of carbon tetrachloride and cyclohexane. Finally *4-amino-α:β-diethyl-β-hydroxydibenzyl* was obtained in colourless needles, m.p. 91–92°. (Found: C, 80·1; H, 8·3%. $C_{18}H_{23}ON$ requires C, 80·2; H, 8·6%.)

4-Amino-α:β-diethylstilbene (23). A solution of 4-amino-α:β-diethyl-β-hydroxydibenzyl (10 g.) in glacial acetic acid (30 ml.) was saturated with hydrogen chloride at room temperature. After 72 hr. it was diluted with conc. HCl (30 ml.) and after a further 72 hr. the thick, rather discoloured precipitate (8·7 g., m.p. 220–230°) was recrystallized (charcoal) from ethanol diluted with 4 vol. of 0·5*N* HCl. *4-Amino-α:β-diethylstilbene hydrochloride* was obtained in colourless needles, m.p. 254–255°. (Found: C, 75·2; H, 7·5%. $C_{18}H_{22}NCl$ requires C, 75·1; H, 7·7%.) The parent *base* regenerated in the usual fashion, crystallized from ligroin (b.p. 40–60°) in sprays of needles, m.p. 96–97°. (Found: C, 86·3; H, 8·4. $C_{18}H_{21}N$ requires C, 86·1; H, 8·4%.)

4-Sulphanilamido-α:β-diethylstilbene (24). Treatment of 4-amino-α:β-diethylstilbene in pyridine solution with 4-acetamidobenzenesulphonyl chloride gave *4-[4′-acetamidobenzenesulphonamido]-α:β-diethylstilbene*. This crystallized readily from methanol in colourless needles, m.p. 207–208°. (Found: C, 69·5; H, 6·6. $C_{26}H_{29}O_3N_2S$ requires C, 69·6; H, 6·3%.)

On hydrolysis with HCl in ethanol, *4-sulphanilamido-α:β-diethylstilbene* was readily obtained. It crystallized from methanol or 95% ethanol in colourless needles, m.p. 180–182°. (Found: C, 70·9; H, 6·4; N, 7·15%. $C_{24}H_{25}O_2N_2S$ requires C, 70·9; H, 6·4; N, 6·9%.)

4-Hydroxy-4′-aminostilbene (27). A solution of 4-hydroxy-4′-nitrostilbene (1·0 g.) in ethanol (15 ml.) and concentrated ammonia (d. 0·880; 16 ml.) was gradually added to a boiling solution of $FeSO_4$ (11·0 g.) in water (75 ml.) and ethanol (150 ml.). The addition completed (1 hr.), the mixture was boiled for 1 hr. After cooling, the precipitated hydroxide was dissolved in dilute HCl, the yellow insoluble portion filtered off and crystallized from hot aqueous 2*N* NaOH. The crystalline sodium salt was collected and the parent phenol regenerated by treatment with dilute acetic acid. The crude *4-hydroxy-4′-aminostilbene* (0·75 g.) was a buff-coloured powder; it was sparingly soluble in most common organic solvents and could not be obtained quite pure. It crystallized from benzyl cyanide in bundles of yellowish plates, m.p. 270–271° (decomp.). (Found: C, 80·1; H, 6·2; N, 7·0%. $C_{14}H_{13}ON$ requires C, 79·6; H, 6·1; N, 6·6%.)

4-Hydroxy-4'-cyanostilbene (28). 4-Cyanophenylacetic acid [Jaeger & Robinson, 1941] (2·0 g.), 4-hydroxybenzaldehyde (1·0 g.) and piperidine (1 ml.) were heated at 140° for 1½ hr. The semi-liquid product was mixed with 50% ethanol, when a yellowish solid was rapidly deposited. This was collected, washed with fresh 50% ethanol, crystallized twice from glacial acetic acid and finally from 70% ethanol: *4-hydroxy-4'-cyanostilbene* was obtained in faintly coloured prisms, m.p. 221–223°. (Found: C, 81·3; H, 5·0; N, 6·4%. $C_{15}H_{11}NO$ requires C, 81·4; H, 5·0; N, 6·3%.)

4-Hydroxy-4'-amidinostilbene hydrochloride (29). This was prepared from 4-hydroxy-4'-cyanostilbene according to the general procedure of Ashley, Barber, Ewins, Newbery & Self [1942]. *4-Hydroxy-4'-amidinostilbene hydrochloride* crystallized from hot water in long yellowish needles, m.p. 316–317° (decomp.). (Found: C, 65·6; H, 5·4; N, 9·9; Cl, 12·5%. $C_{15}H_{15}ON_2Cl$ requires C, 65·6; H, 5·5; N, 10·2; Cl, 12·8%.)

SUMMARY

1. A series of compounds of the stilboestrol type has been prepared and tested *in vitro* for bactericidal activity against staphylococci and streptococci. Several have been found to possess exceptionally high activity, the most active being

$$4\text{-OH.C}_6\text{H}_4\text{.CEt.CEt.C}_6\text{H}_5,$$

which is some 40 times as active as the mould metabolic product citrinin, and as active as chloroxylenol under the conditions employed.

2. A comparison of the bactericidal and oestrogenic activities of these compounds shows that the two actions are not necessarily concurrent, though both probably depend on the ready acceptance by the cell of molecules of the cyclopentanophenanthrene and stilboestrol types.

3. *In vivo* the compounds tested showed little or no activity, possibly on account of their very low solubility.

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Growth-promoting Substances from Bacteriological Filter Pads

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During an investigation of the nutritional requirements of acid-fast saprophytes [Edson & Hunter, 1943], synthetic media were employed, solutions of possible growth stimulants being added aseptically to a basal salt mixture. The methods of sterilization were selected according to the nature and stability of the chemical compounds involved. It soon became evident that the results varied with the mode of preparation and filtration of the test substances. It was shown that solutions, both in water and in saline, were able to extract materials from filter pads in a concentration sufficiently high to stimulate the growth of acid-fast organisms, so that false positive results were sometimes recorded.

MEDIA AND METHODS

The salt mixture, at pH 7·5, was the one described by Edson & Hunter [1943]; the medium and the inoculum were prepared, and growth estimated, as before. Stock solutions of the source of carbon (glucose) and solutions (usually 10 ml. quantities) of substances to be tested were sterilized by filtration through Seitz E.K. or Ford S.B. pads clamped in the glass and steel holders described by Knight & Fildes [1936].

By these methods clear-cut results were obtained, and it was shown that of over 200 different compounds tested, those which were active belonged to four classes: (1) citric acid, (2) α-amino-acids or compounds in which the amino groups were bound in peptide linkages, (3) histamine, (4) ions of calcium and iron.

Growth of Mycobacteria *in media sterilized by different methods*

Anomalous results were first clearly found when solutions of 2:5-diketopiperazine were sterilized by different methods. The results for *Myco. phlei* are set out in Table 1. When growth occurred, it was evident that the anhydride was inactive (flask 5), and that the methods of preparation and sterilization were responsible for the difference in flasks 3 and 4.

Similar examples of 'false' stimulation were encountered when it became necessary to filter the final medium containing other test substances or when the solubility of some compounds precluded the preparation of concentrated stock solutions. Whenever heat-stable substances were added to the medium and autoclaved, no false results were obtained. 'False' stimulation occurred, however, if small quantities of the test substance were added to the basal salt mixture and the whole passed through a 3 or 6 cm. filter pad. Many of the unstable compounds under investigation proved to be sufficiently free from bacterial contamination to permit their use without previous sterilization, and thus it was possible to prove that the stimulation in filtered solutions was 'false'.

Filtration of the basal salt mixture alone through a pad caused stimulation of the growth of acid-fast saprophytes in the presence of a suitable source of carbon. Table 2 shows results of tests in media under different conditions. Growth in a filtered

Table 1. *Effect of method of sterilization of synthetic medium on growth of* Myco. phlei

	Flask no.				
	1	2	3	4	5
Source and treatment of 2:5-diketopiperazine	0·4 ml. of 0·5% autoclaved stock solution added aseptically to autoclaved salt mixture	2 mg. solid added to salt mixture and autoclaved	0·4 ml. of 0·5% filtered stock solution added aseptically to autoclaved salt mixture	2 mg. solid added to salt mixture and filtered	2 mg. unsterilized solid added to autoclaved salt mixture
Growth at 7 days	+	+	Nil	+	Nil

Medium: 20 ml. basal salt mixture containing 1% of glucose and 0·01% 2:5-diketopiperazine. Inoculum, 0·01 mg. Temp. 38°.

copious and was checked by microscopic examination of stained smears, and by subculture of a small portion of surface film on Dorset's egg medium.

Autoclaving of aqueous and saline preparations of 2:5-diketopiperazine (flasks 1 and 2) probably induced hydrolysis to glycyl-glycine, which was subsequently shown to be a growth stimulant. It was basal medium is compared with true stimulation by citrate and by *dl*-alanine.

The stimulant effect was progressively greater as successive 20 ml. portions of salt mixture were passed through the same pad.

One other organism, *Esch. coli* (laboratory stock strain), was also tested, the same basal salt mixture

Table 2. *Growth of saprophytic* Mycobacteria *in different media*

Inoculum: 0·01 mg. seeded in 20 ml. medium. Time, 7 days. Temp. 38°.

	Growth (mg. dry wt.) on				
Organism	Autoclaved basal salt mixture +1% glucose	Filtered basal salt mixture +1% glucose	Autoclaved basal salt mixture +autoclaved citrate (0·01%) +1% glucose	Basal salt mixture +*dl*-alanine (0·01%) autoclaved +1% glucose	Broth controls
Myco. phlei	Nil	6·4	9·6	11·8	46·0
Myco. stercoris	Nil	6·7	9·5	8·0	37·1
Myco. smegmatis	Nil	6·2	9·6	13·2	50·2
Myco. sp. *Karlinski*	Nil	6·5	8·0	8·0	33·4
Myco. sp. *leprous Kedrowsky*	Nil	1·4	Trace	Trace	5·8

being employed, with an inoculum of one drop of a slightly opalescent suspension of washed bacilli. The results were read within 48 hr. and were unchanged after 3 days. No growth occurred on inoculation when glucose or citrate was the source of carbon in the autoclaved basal mixture, but the addition of glucose (1 %) and citrate (0·01 %) to autoclaved salt mixture stimulated heavy growth. When the basal salt mixture containing glucose was filtered through a pad, strong stimulation equal to that obtained by citrate addition was observed. The growth-promoting quality of Seitz filtrates was therefore not confined to species of the genus *Mycobacterium*.

The nature of the growth-promoting substances

The active materials were more easily extracted by water or saline mixture when these were hot than when cold and hence the substances were readily extracted by autoclaving portions of a pad in 20 ml. of basal mixture. Ether and alcoholic extracts were inactive, while tests for citric acid in aqueous extracts were consistently negative within the limits of the micro-tests employed. The pads appeared to consist mainly of asbestos and paper pulp; average ash content, 36·0 %. Sodium silicate, tested as a possible stimulant, was ineffective. Although the water-soluble portion of pad ash (completely ashed in an electric furnace) caused little, if any, growth stimulation, the neutralized acid-soluble (HCl Analar) fraction produced a growth averaging 7·6 mg.

The results indicated that the stimulating inorganic material remained after ashing in a form soluble in dilute HCl. Of the numerous inorganic elements tested [Edson & Hunter, 1943], all were ineffective with the exception of Ca and Fe. It was found that Ca^{++} (50 μg.) and Fe^{+++} (20 μg.), produced growth stimulation approximately equal to that given by pad ash.

The filtered basal medium was very slightly turbid, and its behaviour on heating and cooling was suggestive of the presence of calcium phosphate. A positive test was given with ammonium oxalate, indicating, by comparison against known standards, the presence of about 100 μg. Ca^{++}/20 ml. of medium. The Prussian blue test [Feigl, 1937] revealed a trace of Fe^{+++}, 1–10 μg./20 ml., but the α:α'-dipyridyl test for Fe^{++} was negative. Filter pads, extracted with HCl (Analar Fe-free) gave strong positive tests for Fe^{+++} with thiocyanate reagent, and analysis showed the presence of 250 μg./g., or about 730 μg./6 cm. pad. The pads contained only a trace of Fe^{++} (α:α'-dipyridyl). The iron, however, was not soluble to any great extent in the basal salt mixture. Pad ash gave very strong tests for Fe^{+++} (KCNS) and Ca^{++} (oxalate). It is also of interest to note that acid extracts of pads gave positive tests for copper, determinations based on the diethyl-dithio-carbamate colour reaction [Callan & Henderson, 1929] showing the presence of 10–15 μg./6 cm. pad.

Since previous experiments [Edson & Hunter, 1943] had shown that the growth of acid-fast saprophytes was stimulated by low concentrations of Ca and Fe, it appeared that the presence of these ions was responsible for the results obtained with solutions which had been filtered through the pads. It was further shown that the filtered stock 10 % glucose solutions contained Ca^{++} and traces of Fe^{+++}. In practice 2 ml. of 10 % glucose were added to the salt mixture to give 1 % sugar in the final medium. It was found, however, that 20 ml. of salt mixture, containing 2 ml. glucose solution, gave negative tests for Ca^{++} (by the method used), and that small additions did not supply a sufficiently high concentration of calcium ions to stimulate growth during the period of the test. Similarly the aseptic addition of small volumes, usually less than 0·5 ml., of preparations of test substances (as in Table 1) to autoclaved synthetic medium did not convey sufficient material to affect the growth of *Mycobacteria* under the conditions employed.

DISCUSSION

The findings reported show the possibility of unwittingly adding extraneous materials during filtration of media employed in investigations of bacterial nutrition. Ions of calcium and iron, which have been shown to be growth stimulants for members of the genus *Mycobacterium*, were extracted from filter pads. By comparison of the type of growth stimulated by Ca^{++} and Fe^{+++} separately with that promoted by pad ash and extracts, it was evident that calcium ions were the main stimulating agents and that not enough iron was extracted to affect growth. The growth produced with pad materials and by Ca was relatively unpigmented and somewhat slimy in character, while that with Fe was granular and deeply pigmented. It was noted, also, that whenever the pad filtrates were very slightly turbid, i.e. when the calcium phosphate or traces of fibre which the pads yielded were just visible, the growth of *Myco. phlei* was more profuse. This is analogous with the findings of Bigger & Nelson [1941], on the effect of talc and other insoluble inorganic substances on the growth of coliform bacilli in water.

It is not suggested that the results eliminate the presence of organic growth factors in pads, but in view of the fact that the stimulation obtained in filtered media was of the same order and appearance as that given by pad ash, it seems likely that, for the *Mycobacteria* at least, the ions of Ca and possibly of Fe are the principal growth-stimulating substances extractable from ordinary bacteriological filter pads.

SUMMARY

In a study of the nutritional requirements of various species of the genus *Mycobacterium*, Seitz filtration

was employed for the purpose of sterilizing certain preparations of possible growth stimulants. It was found that Ca^{++} and Fe^{+++} were extracted from filter pads in a single filtration. These ions were growth stimulants for the acid-fast organisms under the conditions used, and their presence in filtrates accounted for anomalous results obtained with some preparations.

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The Mechanism of Coprosterol Formation *in vivo*

2. ITS INHIBITION BY SUCCINYL SULPHATHIAZOLE AND BY CARBARSONE

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The discovery that brain contains a substance, probably identical with or allied to the cerebroside phrenosin, which enables the organism to convert large amounts of cholesterol, added to the diet, into coprosterol [Rosenheim & Webster, 1941], offers a convenient experimental method for investigating the effect on coprosterol formation of drugs which act specifically on the intestinal flora or fauna. In view of the surprising lack of evidence for the theory, advanced on purely theoretical considerations by the early investigators [Bondzynski & Humnicki, 1896; Müller, 1900], and held ever since, that putrefactive intestinal bacteria are responsible for the conversion of cholesterol into coprosterol by direct hydrogenation, we selected in the first instance the sulphonamide derivative succinyl sulphathiazole (S.S.) for investigation. This drug exerts a powerful bacteriostatic action on *Escherichia coli* and other coliform bacteria of the intestine and has the additional advantage of low toxicity, owing to its poor absorption from the alimentary tract.

As the outcome of this investigation, together with other experimental evidence, made it improbable that intestinal bacteria have a share in coprosterol formation, the possibility was considered whether the fauna, and especially the protozoan population, of the intestine rather than its flora was concerned in the process. This possibility was investigated by examining the coprosterol excretion of rats treated with the amoebicidal drug carbarsone (*p*-carbamino phenylarsonic acid). This arsenical also frees the intestinal tract from the flagellate *Trichomonas*, a non-pathogenic parasite occurring in the intestinal canal of man and animals. A drug specifically acting on *Trichomonas* was chosen for the test, since it has been claimed that cholesterol is an essential growth factor for four species of *Trichomonas* [Cailleau, 1937; 1939].

EXPERIMENTAL

Methods

The technique of the animal experiments, the composition of the diet, and the analytical methods used have previously been described [Rosenheim & Webster, 1941]. S.S. was administered by intimately mixing the drug with the diet to the amount of 5% [Welch, Mattes & Latven, 1942]. The S.S. contents of the faeces were determined by the method of Bratton & Marshall [1939]. Carbarsone was given in the drinking water, which consisted of a 0·5% solution of the drug in a 1% solution of sodium hydrogen carbonate in distilled water [Gabaldson, 1936]. *Esch. coli* counts were made by the usual method, using poured MacConkey's plates. The faeces and intestinal contents were examined for *Trichomonas* microscopically and by cultivation in the medium of Dobell & Laidlaw [1926]. The protozoological examinations were kindly made by our colleague, Dr Clifford Dobell.

Experiments with succinyl sulphathiazole

In a preliminary experiment two groups each of three male rats were used. During a period of 3 days they were fed on the stock diet, the experimental group receiving S.S. as well. Bacteriological examination of the faeces on the fourth day showed that *Esch. coli* had been almost eliminated in the experimental group, whilst the faeces of the control group contained 50×10^6 organisms/g.

In the subsequent period both groups were kept for 4 days on a diet of steamed brain (15 g./day/rat), the experimental group receiving S.S. as before. The faeces of this group were again found to be free from *Esch. coli*, the control group containing 80×10^6 organisms/g. Both groups lost in weight, the experimental group losing twice as much as the control groups. The analytical results are summarized in Table 1.

Table 1. *Influence of the addition of succinyl sulphathiazole to a diet of brain, on the faecal excretion of coprosterol and of* Esch. coli, *by the rat*

Period	Duration of period (days)	Total wt. of food consumed (g./day)	Total body weight: Initial (g.)	Final (g.)	Loss (g./day)	Total wt. of moist faeces (g.)	Faecal excretion, during last 24 hr. of period, of: *Esch. coli* (no./g. of moist faeces)	Succinyl sulphathiazole (g./100 g. of moist faeces)	Succinyl sulphathiazole (g./100 g. of dry faeces)	Faecal excretion of sterols during whole period: Total (g.)	Coprosterol (g.)	Coprosterol (% of total)
Control	3	45	568	509	19·7	40·7	80×10^6	None	None	3·39	2·54	74·9
Experimental	3	45	534	418	38·7	42·7	1×10^3	4·6	13·6	2·34	Trace	<1

The addition of S.S. to the brain diet produced almost complete inhibition of coprosterol formation, whilst the coprosterol excretion of the control group on brain diet alone was of the same order as in our previous experiments [Rosenheim & Webster, 1941]. Pure cholesterol, m.p. 148–149°, was recovered from the digitonides obtained in the estimation of the sterols in the faeces of the experimental group, whilst those of the control group yielded coprosterol, m.p. 100–101°, in typical needles.

The administration of S.S. was stopped on the fifth day and the animals were put on a diet of brain alone, for 4 days. Only one of the rats survived this period, the high mortality rate probably being due to the increased toxicity of the drug when given in a diet low in protein [cf. Smith, Lillie & Stohlman, 1941; Kapnick, Lyons & Stewart, 1942]. Examination of the faeces of the surviving animal showed, however, that in spite of the return, in enormously greater numbers, of coliform bacteria (100×10^6/g.), the coprosterol content amounted to only 7 % of the total sterols.

In the next experiment the high mortality rate was avoided by substituting for brain the standard diet supplemented by cholesterol (160 mg./rat/day) and cholesterol-free brain powder (1·6 g./rat/day). The fact that the specimen of brain powder [Rosenheim & Webster, 1941] used had been prepared 6 years ago and had retained its full activity, incidentally proves the stability of the factor contained in it.

Two groups of three male rats each were used, the experimental group receiving S.S. for a preparatory period of 3 days. The experimental period, with the additional supplement of brain powder and cholesterol + S.S., lasted 4 days, the faeces of the last 3 days being collected for analysis. During this and part of the subsequent period the faeces were semifluid and unavoidable losses occurred in their collection. After 4 days the administration of S.S. was stopped and the animals were kept on the standard diet and brain powder + cholesterol for 7 days.

The results (Table 2) were practically identical with those of the previous experiment, in which brain was the sole food. The animals remained in good health, however, and during the short experimental period they actually increased in weight at the same rate as the control rats. In this connexion it is significant that the S.S. content of the faeces

Table 2. *Influence of the addition of succinyl sulphathiazole to a normal diet supplemented with cholesterol and brain powder, on the faecal excretion of coprosterol and of* Esch. coli, *by the rat*

Period	Duration of period (days)	Rats in group	Total wt. of food consumed (g./day)	Total body weight: Initial (g.)	Final (g.)	Increase (g./day)	Total wt. of moist faeces (g.)	Faecal excretion, during last 24 hr. of period, of: *Esch. coli* (no./g. of moist faeces)	Succinyl sulphathiazole (g./100 g. of moist faeces)	Succinyl sulphathiazole (g./100 g. of dry faeces)	Faecal excretion of sterols during whole period: Total (g.)	Coprosterol (g.)	Coprosterol (% of total)
Control	3	3	34	486	505	6·3	13·0	17×10^6	None	None	1·02	0·77	75·5
Experimental	3	3	31	463	480	5·7	16·6	1×10^3	7·5	27·7	0·62	Trace	<1
Post-experimental	6	3	33	480	518	6·3	16·1	32×10^6	None	None	1·05	Trace	<1

on the last day of the experimental period was nearly double that of the faeces on the corresponding day in the preceding experiment (see Table 1), in which brain was the exclusive diet. A greater absorption of the drug from the intestinal tract may thus explain the higher mortality on the brain diet.

The persistence of the inhibitory effect on coprosterol formation was again shown, in spite of the return of *Esch. coli* in the faeces after the withdrawal of the drug. The duration of this inhibitory period has not yet been studied in detail, but in one experiment it was found that the coprosterol excretion was still *c.* 15% below the normal level after 3 weeks on the standard diet.

Experiments with carbarsone

In a preliminary experiment a male rat, weighing 220 g., was taken at random from the Institute's colony. On microscopical examination of its faeces, the animal was found to be heavily infected with *Trichomonas muris*. A feeding test with steamed sheep's brain showed a coprosterol excretion of 78% of total sterols. The rat then received 45 mg./day of carbarsone whilst on the standard diet for 2 days. During the next 5 days the standard diet was replaced by steamed sheep's brain (25 g.) containing bone meal (1 g.) and the dosing with carbarsone was continued. The rat received 210 mg. of carbarsone, i.e. 42 mg./day. At the end of this period the faeces were free from *Trichomonas*, but showed a high count of *Esch. coli* (100×10^6/g.). The faeces of the last 4 days were collected and analyzed. The sterols consisted of practically pure cholesterol containing only traces of coprosterol.

This striking inhibitory effect of carbarsone on coprosterol formation was confirmed by the subsequent experiments, which were carried out on individual animals in order to ensure adequate control in the collection of faeces for bacterial and protozoological examination. Male rats of the average weight of 270 g. were kept in separate cages with coarse wire-grid bottoms. For the first 5 days their diet consisted of a mixture, in equal parts, of the standard diet and steamed sheep's brain. All the rats were found to be infected with *Trichomonas* and bacteriological examination of the faeces showed the usual high count of *Esch. coli* at the end of this period. During the carbarsone period the drug was administered for 1 day in the drinking water, whilst the rats were on the standard diet. During the following 5 days they received known amounts of carbarsone (see Table 3) in their food, consisting of the mixture of steamed brain and standard diet described above. At the end of the experimental period the rats were in good condition. The faeces were found to be free from *Trichomonas*, whilst the *Esch. coli* counts were as high as before. The results of four experiments of this type, in which the faeces of the last 3 days were collected and analyzed, are given in Table 3.

The figures in the last column of Table 3 demonstrate conclusively the inhibitory effect of carbarsone on coprosterol excretion, which has been reduced to the merest traces even in the rat in which, before the administration of carbarsone, coprosterol accounted for as much as 90% of the total sterols excreted.

Attempted bacterial hydrogenation of cholesterol and cholestenone

The possibility that the failure of previous attempts to effect coprosterol formation by intestinal bacteria *in vitro* may have been due to the absence in the media used of an essential growth factor, led us to investigate their action on cholesterol and cholestenone in the presence of brain, which favours coprosterol formation *in vivo*.

(1) *Cholesterol.* (*a*) Flasks containing minced sheep's brain (10 g.) inoculated with faeces (1 g.) from rats fed on brain, were incubated in anaerobic jars at 37° for 8 and 16 days respectively. The unsaponifiable matter was prepared in the usual

Table 3. *Influence of the addition of carbarsone to a normal diet supplemented with brain, on the excretion of coprosterol by the rat*

Period	Duration of period (days)	Rat no.	Carbarsone intake (mg./day)	Total wt. of moist faeces (g.)	*Trichomonas* infection	Faecal excretion of sterols during whole period: Total (g.)	Coprosterol (g.)	Coprosterol (% of total)
Control	3	1	0	10·0	+ + +	0·83	0·62	74·7
	3	2	0	6·2	+ + +	0·81	0·68	83·9
	3	3	0	7·7	+ + +	0·82	0·74	90·3
	3	4	0	6·1	+ + +	0·65	0·52	80·0
							Average	82·2
Experimental	3	1	69	10·5	None	0·73	Trace	<1
	3	2	64	8·4	None	0·69	Trace	<1
	3	3	63	12·1	None	0·88	Trace	<1
	3	4	60	7·6	—*	0·64	Trace	<1

* Not examined.

way for the estimation of total and saturated sterols by the gravimetric digitonin method. The cholesterol content of the brain was 2·6 % and the mixture in the control flask contained 37 mg. of coprosterol, derived from the added faeces. At the end of the experiment 36 mg. (± 7) of coprosterol were found in the incubated mixtures, showing that no conversion of cholesterol into coprosterol had taken place. (*b*) In another set of experiments 10 g. portions of minced sheep's brain, ground up with 20 ml. of glucose broth, were sterilized and inoculated with 5 ml. of a 24-hr. culture of mixed anaerobic bacteria. This culture was obtained from the caecal contents of three rats excreting large quantities of coprosterol on a diet of sheep's brain. After 17 days' incubation under anaerobic conditions the brain mixtures yielded 2·55 % and the control 2·65 % of cholesterol. No coprosterol had been produced.

(2) *Cholestenone.* (*a*) Suspensions of finely powdered cholestenone (200 mg.) in glucose broth (50 ml.) were sterilized and inoculated with a fresh mixed culture of intestinal bacteria (see above). No sterols precipitable by digitonin were obtained from these mixtures after anaerobic incubation for 17 days. It has previously been shown [Mamoli, Koch & Teschen, 1939] that cholestenone was not attacked by a pure culture of *B. putrificus* under the conditions in which the keto group and ethenoid linkage of testosterone, androstenedione, etc. were reduced. (*b*) Flasks containing 10 g. portions of sheep's brain, inoculated with faeces as above, and intimately mixed with a solution of 0·2 g. cholestenone in cetyl alcohol, were incubated anaerobically for 8 and 16 days respectively. The average amount of coprosterol recovered, 33 mg. (± 1), was slightly less than the amount (37 mg.) derived from the added faeces. These negative results agree with those of similar experiments by Diels [1937].

DISCUSSION

It is tempting to associate the striking inhibitory effect of succinyl sulphathiazole on coprosterol formation with the simultaneous disappearance, caused by the drug, of *Esch. coli* and coliform bacteria from the intestine. The conclusion that these bacteria are therefore directly responsible for coprosterol formation is, however, fallacious and disproved by the fact that the inhibitory effect persists in spite of the immediate return, in enormously increased numbers, of *Esch. coli* and coliform bacteria after the withdrawal of the drug.

The concept that coprosterol originates in the intestine by bacterial hydrogenation of cholesterol arose at a time when the stereochemical differences in the constitution of the reduction products of cholesterol were entirely unknown. Since then it has been recognized that, under all known conditions, the hydrogenation of the Δ^5 ethenoid linkage of cholesterol leads to the *trans*-decalin derivative (β)-cholestanol and that coprosterol, the corresponding *cis*-decalin derivative, can be obtained only by indirect methods. In ignorance of these facts, the early investigators linked up coprosterol formation in the intestine with the activity of 'putrefactive' bacteria, an apparently obvious interpretation of the facts known to them. Although this simple solution of a complex problem has not been supported by any convincing experimental evidence, it is still current. As an argument against the bacterial origin of coprosterol the fact may be adduced that bacteria are devoid of cholesterol, a sterol found in animal cells only. Moreover, bacteria and the primitive unicellular algae, Myxophyceae [Carter, Heilbron & Lythgoe, 1939], are the only cellular organisms in which sterols of any kind are absent.

Evidence is accumulating in favour of the view that the chemical changes occurring in coprosterol formation proceed in two stages, consisting in a primary oxidation of cholesterol to cholestenone followed by reduction of the latter to the *cis*-decalin derivative coprosterol [Rosenheim & Webster, 1943]. Whether these reactions are brought about by the enzymic activity of the intestinal mucosa or by other agencies is unknown. Our results show that intestinal bacteria in any case are unable to reduce cholestenone to coprosterol *in vitro*, even in the presence of the hypothetical growth factors, contained in brain, which favour coprosterol formation *in vivo*. In this connexion the recent work on the bacterial reduction and oxidation of steroid sex hormones, carried out mainly in Butenandt's laboratory, acquires particular importance. Some of the incidental observations recorded in these researches have a direct bearing on the coprosterol problem, since they led to the conclusion that the susceptibility of steroids to bacterial attack is dependent on the length of the side chain: cholesterol, cholestenone and cholestanone, possessing the intact side chain of eight carbon atoms, are recovered unchanged when subjected to the action of certain dehydrogenating or hydrogenating bacteria respectively, under the same conditions which bring about the oxidation or reduction of the functional groupings of steroid sex hormones possessing a shorter or no side chain [Mamoli & Schramm, 1938; Mamoli, 1938; Mamoli *et al.* 1939]. It is therefore not surprising that all attempts to convert cholesterol into coprosterol by bacterial action *in vitro* should have failed, and it appears unlikely that cholesterol is concerned in bacterial metabolism.

If we accept the view that bacteria have no share in coprosterol formation, it seems attractive to consider as an alternative the possible role of the intestinal fauna, and especially of protozoa, in this

process. Various species of flagellates belonging to the genus *Trichomonas* are non-pathogenic inhabitants of the intestinal tract of man and animals, so that the caecums and intestines of rats 'are often swarming with *Trichomonas*' [Wenyon, 1926]. A possible connexion between these protozoa and cholesterol metabolism is suggested not only by the fact that four species of *Trichomonas* require cholesterol as an essential growth factor [Cailleau, 1937; 1939], but also by various other considerations. Thus, infection with *Trichomonas* of babies up to 1 year is rare [Faust & Headlee, 1936] and their faeces, like the meconium, are devoid of coprosterol [Flint, 1862; Müller, 1900; Fox & Gardner, 1925]. When the consumption of a mixed diet at later stages of their development favours the chances of infection by the flagellates, coprosterol appears in their excreta. Further, an exclusive milk or meat diet reduces both the trichomonad infection [Hegner, 1923; 1924] and coprosterol excretion [Müller, 1900; Dorée & Gardner, 1908].

Attractive as this suggested correlation between protozoa and coprosterol formation seemed to be, and in spite of its apparent confirmation by the results of the above-described experiments with the arsenical drug carbarsone, such a correlation appeared to be fortuitous. On further investigation, it was found that rats, freed from *Trichomonas* by carbarsone treatment, excreted a considerable amount of coprosterol in the period following the stoppage of the drug. On the other hand, administration of succinyl sulphathiazole caused the evacuation of large numbers of *Trichomonas* in the faeces without freeing the rats from the infection, but they excreted only small amounts of coprosterol for some time after cessation of the treatment. It is realized that our exploratory experiments in this direction are liable to various interpretations. The results so far obtained, however, indicate that the participation of protozoa in coprosterol formation is at least as unlikely as that of bacteria.

It is equally difficult to relate the interference with cholesterol metabolism exhibited by carbarsone and succinyl sulphathiazole with the inhibitory effect of the latter drug on the synthesis of the essential vitamins, biotin and folic acid, by intestinal bacteria [Nielsen & Elvehjem, 1942; Welsh & Wright, 1943], especially as a possibly similar effect of carbarsone has not yet been investigated.

The unexpected fact thus emerges that two drugs of dissimilar chemical constitution, one of which acts specifically on *Esch. coli* and not on protozoa and the other on Protozoa but not on *Esch. coli*, have in common an inhibitory effect on coprosterol formation, which does not seem to be due to their direct action on either the bacteria or protozoa concerned. The mechanism of this inhibitory action remains so far unexplained.

SUMMARY

1. The bacteriostatic action of succinyl sulphathiazole on intestinal coliform bacteria was found to be concurrent with a complete inhibition of coprosterol formation. The apparent correlation, however, between *Esch. coli* and coprosterol formation is fortuitous and the available evidence makes it improbable that intestinal bacteria have any share in the process.

2. The possibility that the fauna, and especially the protozoan population, of the intestine rather than its flora is concerned in coprosterol formation was investigated by examining the coprosterol excretion of rats treated with carbarsone (*p*-carbamino phenylarsonic acid). This amoebicidal drug frees rats from *Trichomonas muris* infection and was found also completely to inhibit coprosterol formation. Exploratory experiments, however, indicate the absence of a relationship between protozoa and coprosterol formation.

3. The complexity of the problem is made apparent by this interference with cholesterol metabolism, shared by an antibacterial and an amoebicidal drug, which seems to be unconnected with their action on either bacteria or protozoa and which remains so far unexplained.

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A Micro-method for the Estimation of Vitamin B_1

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A micro-method for the estimation of vitamin B_1 has been described by Atkin, Schultz & Frey [1939]. It is based on the measurement of the rate of fermentation of sugar by baker's yeast, using a Warburg or similar apparatus, and is sensitive to 0·005 μg. of vitamin B_1. The method described below is based on the thiochrome technique and is of similar sensitivity. It was developed during work on the distribution of vitamin B_1 in the wheat grain and has been applied successfully to small quantities (1–50 mg.) of cereal products [Hinton, 1942].

EXPERIMENTAL

The method is based upon the optical design of the Spekker Photoelectric Fluorimeter. In this instrument, an image of the mercury discharge tube is brought to a focus at a point opposite the photo-cell, the focused beam normally passing through the test solution contained in a large glass cell. In the micro-method here described, the glass cell is replaced by a suitable holder by means of which a capillary tube containing the test solution is placed in the image, which measures 2 × 20 mm. approximately in the focal plane, and thus receives the full irradiation transmitted by the system (Fig. 1).

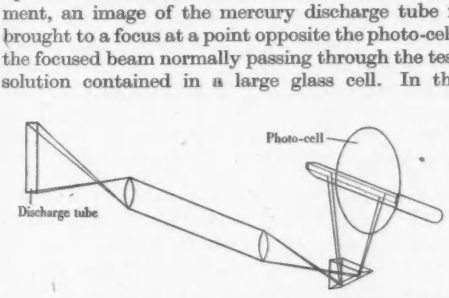

Fig. 1. Diagram showing optical design of the Spekker Photoelectric Fluorimeter and position of capillary-tube test cell used in the micro-method.

The Spekker Fluorimeter was used in conjunction with a Cambridge Short-Period Galvanometer of sensitivity 3000 mm./μA., and resistance 196 ohms, which was used at full sensitivity.

Procedure

The strength of ferricyanide to produce maximum fluorescence in the test solution is first ascertained by the procedure summarized below. With our cereal extracts this was found by testing a range of strengths from 0·05 to 0·5 %, in steps of 0·05 %. For standard aneurin solutions the best concentration was 0·005 %.

The pipette to be used for the test solution is first washed out with the solution, in readiness. 0·1 ml. of 30 % NaOH is measured into a 2 × $\frac{3}{8}$ in. tube, 0·02 ml. of the appropriate ferricyanide solution run in on top and left undisturbed. 0·1 ml. of the test solution is then measured out and, immediately before this is added to the tube, the NaOH and ferricyanide are mixed by shaking. The test solution is run in and the whole again mixed by shaking. Oxidation is carried out in this way with each tube in a series which, in order to check the calibration curve, includes three standard aneurin solutions of different strengths. 0·2 ml. of water-saturated isobutanol is then added to each tube, and extraction carried out by stirring for 30 sec. The tubes are allowed to stand for 10–15 min. to allow separation of the layers, those containing cereal extracts being centrifuged for 3–4 min. at 1500 r.p.m. to improve the separation. The isobutanol layer is drawn up directly into the capillary tube for measurement in the fluorimeter. A no. 39 Wratten filter was used in front of the right-hand photo-cell (Fig. 1).

The calibration curve was prepared by the same procedure, using solutions containing from 0·001–0·2 μg. aneurin/0·1 ml.

Examination of the procedure

Accuracy of the method. Standard aneurin solutions in $N/20$ HCl were found to be stable until cloudiness and growth developed. In most cases they were stable for several months. Cereal extracts were made with $N/5$ HCl [Nicholls, Booth, Kent-Jones, Amos & Ward, 1942] applied on a micro scale.

In the case of solutions of pure aneurin the extreme variations between replicates were $\pm 20\%$ at concentration of aneurin in the region of 0·001 μg., decreasing to $\pm 3\%$ at 0·05 μg.

It should be noted that attention was paid to the possibility of decomposition of thiochrome by light during the preparation of the thiochrome solution, especially in the case of the smallest quantities. Readings obtained when the operations were carried out near a window in the full daylight of a clouded sky did not, however, differ significantly from those carried out in very weak artificial light (1–2 candle power). The only precaution taken, therefore, was to shade the operations from sunlight or from very bright sky light.

The capillary test-cell and holder. Greatest sensitivity was obtained with a glass tube of oval section and 1 × 3 mm. bore. For good results certain details required attention: the holder (Fig. 2) was made so that the angle which the

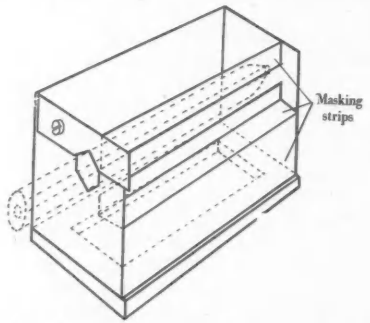

Fig. 2. Holder made from sheet metal with $\frac{1}{8}$″ brass base to fit the cell carrier of the Spekker Photoelectric Fluorimeter and hold the capillary-tube test cell at the correct angle and position.

oval section made with the light beam could be adjusted to the optimum and the tube adjusted squarely in the beam; fluorescence from the glass wall was masked off; adjustments were made to the lamp-house, sliding carrier, etc. of the fluorimeter to make the whole as stable as possible; additional shielding of the photo-cells was provided and great changes in illumination of them during a period of use was avoided.

The length of the tube was 8 cm. To fill it a rubber 'policeman', slipped right down over the end of the tube, is slid carefully upwards whilst the point dips below the surface. The contents are ejected by the reverse movement. On each occasion before the tube is placed in position for measurement the outside should be cleaned by moistening with ethanol and polishing with a cloth. A considerable amount of isobutanol remains on the inside of the tube after it is emptied and the tube should therefore be rinsed with two lots of the solution to be measured. This is done by drawing up a drop of the solution and running it along the tube. The same tube was used for measurement of blank, standard and unknown, and it should be marked and always placed in the holder the same way up.

Standard of fluorescence. As a permanent standard, a crystal of fluorite was used, which was mounted in a holder and masked by a diaphragm to give a fluorescence intensity of the same order as a solution of quinine sulphate of 0·5 μg./ml.

Expression of results. The figures given in the text to illustrate each point are expressed in a relative form. They were expressed in terms of aneurin found on the basis of the fluorescence reading, and then recalculated for each series, the highest yield in the series being taken as 100.

Optimum volumes of reagents. To attain maximum sensitivity, the amount of isobutanol was kept at a minimum. The tube required 0·12 ml., and a total of 0·2 ml. provided enough excess to give the necessary two rinsings. Isobutanol saturated with water was used. 0·1 ml. was chosen as a convenient quantity of test solution, and 0·1 ml. of NaOH gave a total volume most suitable for the subsequent extraction. The smallest quantity of potassium ferricyanide solution which could be handled with confidence is 0·02 ml., and this amount was used in all cases. It was found most satisfactory to keep the volume constant in each case and to adjust the concentration of the solutions as necessary. The reactions were carried out in test-tubes $2 \times \frac{3}{8}$ in.

Handling of these small volumes was facilitated by constructing special pipettes to hold the quantities required, the capillary tube being drawn off slightly and the tip bent round and ground (Fig. 3). The solutions are blown out on to the side of the tube and can be placed just where required.

Fig. 3. Micro-pipette.

Methanol was omitted, as, under these conditions, it seriously affected the partition of the thiochrome in the two phases. Jansen [1936] used methanol because of its protective action towards thiochrome, which is thus made less sensitive to excess ferricyanide. The methanol, however, increases the proportion of thiochrome remaining in the aqueous phase; this effect is not serious when the volume of isobutanol is many times greater than the aqueous phase. In the present case, the volumes of the two phases are equal and the serious effect of the methanol on the final fluorescence is seen from the following figures:

Methanol (ml.)	Nil	0·01	0·04	0·08
Reading	100·0	95·0	81·5	54·0

These results were obtained by carrying out the oxidation in the absence of methanol, which was added finally with the isobutanol. 0·08 ml., which corresponds to about 30% of the reaction mixture, causes an excessive reduction in sensitivity. The aim was to attain maximum sensitivity, but some reduction could be accepted if tolerance of the thiochrome towards excess ferricyanide was thereby obtained. (Tolerance in this paper is defined as the range of concentration of ferricyanide over which the same fluorescence reading is obtained for a fixed amount of aneurin.) The following figures illustrate the tolerance obtained, with 0·2 μg. standard aneurin solutions, by the addition to the

reaction mixture of 0·02 ml. of methanol, which produces a permissible drop in sensitivity:

Strength of ferricyanide (%)	0·003	0·006	0·012
Reading	89·0	100·0	100·0
Strength of ferricyanide (%)	0·025	0·05	0·1
Reading	100·0	97·0	93·5

There is a tolerance range of about 8 times. Without methanol, the tolerance range is reduced to twice:

Strength of ferricyanide (%)	0·0006	0·0012	0·0025	0·005	0·01
Reading	47·5	82·0	99·0	100·0	95·0

As 50 times more ferricyanide is required for a cereal extract than for the standard aneurin solutions, the slight increase in tolerance noted above was not worth considering. In Jansen's experiments he used about 60% methanol in the reaction mixture and reported a range of 500 times.

It is noted from the two series of figures that the optimum strength of ferricyanide is 10 times greater when methanol is present. This is because the methanol enters into the reaction, inactivating the ferricyanide. With 0·1% ferricyanide the following figures were obtained:

Methanol (ml.)	0·01	0·04	0·08
Reading	100·0	88·7	6·8

This effect is much greater than the effect on the thiochrome partition. With 0·5% ferricyanide solution, the figures were:

Methanol (ml.)	0·01	0·04	0·08
Reading	89·5	100·0	77·0

In light of the effect of the methanol on partition, this indicates a reversed effect which is due to reduction of the ferricyanide concentration towards the optimum by the action of the methanol.

The inactivation of the ferricyanide proceeds slowly, and the above results were obtained by mixing the NaOH, ferricyanide and methanol together and allowing the mixture to stand some minutes before adding the aneurin solution. The time effect is illustrated by the data obtained with 0·05% ferricyanide:

	Reading
Aneurin (0·2 μg.) added immediately on mixing	100·0
Aneurin (0·2 μg.) added after 1 hr.	50·0

Aneurin (0·15 μg.) added after time intervals as shown:

Min.	3	8	13	18	23	28
Reading	100·0	98·5	96·8	91·5	88·0	78·0

Again, if a stronger ferricyanide solution is used, the effect is first to increase the fluorescence, which is due to reduction in ferricyanide towards the optimum. The following data were obtained with 0·1% ferricyanide:

	Reading
Aneurin (0·2 μg.) added immediately on mixing	89·5
Aneurin (0·2 μg.) added after 45 min.	100·0

The strength of the NaOH solution has an effect on the speed of this reaction, e.g. with 0·1% ferricyanide and 0·04 ml. methanol, and after the same time of standing these figures were obtained with 0·15 μg. aneurin:

NaOH (%)	30	40	60
Reading	100·0	54·0	10·7

Again, with a stronger solution of ferricyanide and the same amount of methanol, the effect is reversed:

Reading with 0·5% ferricyanide	76·0	89·0	100·0

Optimum strength of ferricyanide. Since it is not practicable to add sufficient methanol to affect the tolerance appreciably, the strength of ferricyanide needs adjusting for each type of material tested, to obtain maximum fluorescence. For standard aneurin solutions the optimum strength is 0·005%.

Aneurin (μg.)	Ferricyanide (%) 0·0005	0·001	0·002	0·003	0·004	0·005
0·15	66·3	77·8	91·2	95·5	97·5	100·0
0·01	—	61·6	96·0	86·0	—	100·0
0·005	—	80·0	—	75·0	—	100·0

Aneurin (μg.)	Ferricyanide (%) 0·006	0·007	0·008	0·009	0·01
0·15	97·0	95·0	94·5	93·5	88·0
0·01	—	—	—	—	—
0·005	—	90·0	—	—	—

The optimum strength for various cereal extracts is shown below:

	Ferricyanide (%) 0·05	0·10	0·15	0·20
No. 1 extract	63·0	90·5	100·0	97·0
No. 2 extract	59·0	88·5	99·5	100·0
No. 3 extract	41·2	67·0	91·0	94·0

	Ferricyanide (%) 0·25	0·30	0·35	0·40
No. 1 extract	96·0	94·5	93·0	92·5
No. 2 extract	99·5	96·5	89·0	91·5
No. 3 extract	98·5	100·0	99·0	99·5

A ferricyanide solution of 0·005% is unstable if exposed to strong light, decomposing in 30 min. or so. Protected from the light, it has been kept for 1 week and may be stable for longer. It was preferred to make a fresh solution by diluting a stock solution of 0·5% each day, and to keep it shaded from daylight.

Optimum strength of NaOH. Solutions varying from 7 to 60% have been tested with varying strengths of ferricyanide. The following figures illustrate the results and indicate that 30% is the most satisfactory:

NaOH (%)	20	25	30	35	40
Reading	89·0	96·2	100·0	96·2	89·0

Order of mixing the reagents. The following figures indicate the degree of destruction of 0·2 μg. of aneurin when it is mixed with the NaOH and allowed to stand varying times before the ferricyanide is added:

Ferricyanide added after (min.)	0	3	6	9	12	15
Reading	100·0	70·0	47·5	34·0	25·4	14·3

Jansen mixed the aneurin solution and ferricyanide first, adding the NaOH immediately afterwards. Greater fluorescence is obtained, however, by adding the aneurin solution to the mixture of NaOH and ferricyanide:

0·2 μg. of aneurin added to ferricyanide	83·5	81·3	77·2
0·2 μg. of aneurin added to mixture of NaOH and ferricyanide	100·0	100·0	100·0

The mixture with NaOH is unstable, when the ferricyanide concentration is of the order of 0·005%. The following method of mixing the reagents was therefore adopted: 0·1 ml. of NaOH is placed first in the bottom of the tube. 0·02 ml. of ferricyanide is then pipetted on to the side of the tube just above the surface of the NaOH, so that it runs down and floats on the surface of the solution. This partition is not disturbed until the test solution is measured out and is ready for adding. With the pipette in one hand, the NaOH and ferricyanide are mixed by shaking the tube, the test solution immediately run in and the whole shaken again. All the reagents are thus mixed together within 30 sec. The times shown in the results below are times elapsing between floating the ferricyanide on the NaOH, and mixing with addition of aneurin solution:

Time elapsing (sec.)	20	120	180	240
Reading	99·7	99·7	98·0	100·0

Once this stage has been reached, the addition of, and extraction with, isobutanol can be carried out within any reasonable time. In the following series the stated times elapsed between carrying out the oxidation and extraction with isobutanol.

Time elapsing (min.)	0	5	10	15	20	25
Reading	95·3	100·0	95·3	95·5	95·3	99·0

Extraction with isobutanol. With these small volumes, bubbling a gas through the liquid is not a successful method of mixing the two phases. Stirring was found to be quite satisfactory. A small glass stirrer made from glass rod drawn out to a diameter of about 1 mm. and shaped into an elongated loop 1 cm. long was fastened directly in the chuck of a small laboratory motor. A frictional brake gave the smooth speed control necessary. With standard aneurin solutions, high speeds, 1000 r.p.m. or more, were necessary to break up the phases completely, but with cereal extracts much slower speeds, 200 or 300 r.p.m., were sufficient and essential, since high speeds produced a fine emulsion which could not be broken down by simple methods. Extracts from some products, e.g. wheat germ, emulsified more readily than others, e.g. endosperm; the lowest speed which sufficed to break up the phases was never exceeded. The following experiment, in which the solution was oxidized in bulk and portions taken for extraction, shows that time of mixing is not critical:

Time of mixing (sec.)	5	10	20	40	60
Reading	95·7	99·5	97·5	100·0	99·0

As a measure of safety, 30 sec. was adopted as standard. The two layers are left in the original tubes, the isobutanol being drawn off directly into the capillary test cell. With cereal extracts the isobutanol layer was cleared by centrifuging at about 1500 r.p.m.; this was not necessary with standard aneurin solutions. Within reason, the time elapsing between extraction and measurement in the fluorimeter is not important:

Measured after (min.)	0	5	10	15	20	25
Reading	95·0	97·5	99·0	99·0	100·0	97·0

Calibration curves. Calibration curves have been prepared with different filters in front of the right-hand photocell; three of these are reproduced in Fig. 4. The shape of the curve is dependent upon the transmission of the filter and the quality of the light emitted by the fluorescent standard. With Wratten filter no. 47 (curve not given) the maximum transmission is in the region a little above the thiochrome fluorescence. For this reason, the photo-cell will not respond much below 0·005μg. The Hilger no. 6, which has a similar but broader transmission, produces a similar shaped curve, *A* in Fig. 4, and as it is much less

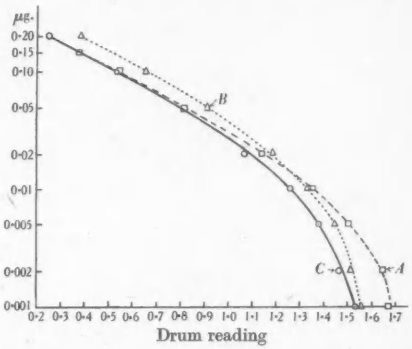

Fig. 4. Calibration curves showing drum reading against micrograms of aneurin measured with: *A*, Hilger filter no. 6; *B*, Wratten filter no. 39; *C*, no filter.

dense, allows the photo-cell to respond to 0·001μg. The lowest measurements, however, come on an insensitive part of the drum, and with this filter a group of calibration curves shows considerable spread below 0·01μg. The Wratten no. 39, though of overall density greater even than that of no. 47, transmits well in the region of thiochrome fluorescence and allows response at 0·001μg. The shape of the curve, *B* in the figure, is closely similar to the shape of the unfiltered curve *C*. This filter also shifts the lowest reading slightly on the drum and a group of curves showed no serious spread over the whole range. On these grounds, no. 39 was judged to be the most suitable filter so far used.

Each curve shown in Fig. 4 is the average of a group of six to eight complete curves. It is desirable to deal with groups of curves in this way as, in common with other photo-electric colorimeters, the Spekker may exhibit a slight shift in the whole curve from day to day. This is particularly the case when it is used as described here, and for this reason it is advisable to put one or two aneurin standards in with each run of estimations. When the capillary test cell containing the thiochrome solution has been placed in position, the photo-cells must be balanced without delay, as the thiochrome is then unstable and the readings fall continuously, especially with solutions of 0·1μg. of aneurin and above. There is ample time, however, to carry out the balancing precisely, as no significant change in the reading occurs in 30 sec.

SUMMARY

1. A micro-technique for the estimation of vitamin B_1 has been developed. The method is based on the determination of thiochrome with the Spekker Fluorimeter. A special cell is used and a precise plan must be followed with the different

reagents normally used in the conversion to thiochrome.

2. In solutions of pure aneurin (or in cereal extracts) the method is accurate to ±20% at concentrations in the region of 0·001 μg., the accuracy increasing to ±3% at 0·05 μg.

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Application of the Millon Reaction to the Determination of Chlorophenols in Body Fluids and Tissues

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The versatile antibacterial usefulness of halogenated phenols makes valuable a specific micro-method for their determination in body fluids and tissues [Zondek, 1942]. The present paper describes a semi-micro-method for *p*-chloro-*m*-xylenol (referred to as *CX*) and *p*-chloro-*m*-cresol (referred to as *CC*) in the presence of phenol, e.g. in urine. Patients given *CX* may excrete this substance in concentrations up to 50 mg./100 ml. The halogenated phenol is allowed to react with Millon reagent at room temperature, the coloured substance so formed being extracted with ether and thus obtained in crystal-clear solution suitable for photometric measurement. Phenol itself under these conditions gives a coloured substance which is only sparingly soluble in ether. The method is therefore highly specific.

Several investigators have used the Millon reaction for the determination of tyrosine, e.g. Folin & Ciocalteu [1927]. The present successful extension of such a method to at least two selected chlorophenols suggests that further investigation would render possible a more general application of the Millon reaction to analytical methods, in which the conventional procedures for the estimation of phenols are often inadequate.

EXPERIMENTAL

Reagents. Reagent 1: A mixture of 50 ml. of a 20% solution of mercuric acetate and 30 ml. of 25% (v/v) HNO_3 ($d. = 1{\cdot}42$).

Reagent 2: A 1 : 4 diluted solution of 100 g. of mercury in 200 g. of HNO_3 (Millon reagent).

Standard solutions: 1 g. of *CX* or *CC*, twice recrystallized from ethanol and dried *in vacuo*, is dissolved in 5 ml. N NaOH and diluted with H_2O to 1 l. In amber bottles the solutions are constant for at least 1 month.

A. *Procedure with aqueous solutions*

Determination of CX. To 15 ml. of test solution in a test-tube of *c.* 30 ml. capacity are added 2 ml. of reagent 1 and then, if no precipitate has formed, 1 ml. of reagent 2. If a precipitate forms, filter and carry out the test on a measured portion. The mixture is kept at room temperature for 30 min., cooled in ice-water, and 5 ml. of chilled ether added. The colour is brought into the ether phase by shaking, and the test-tube immediately stoppered. Within the next hour the ether extract is transferred to a 0·5 cm. cuvette covered with a glass slide. The extinction value (*E*) is determined in a Pulfrich photometer with filter S 43. The concentration of the unknown is read by reference to a standard curve prepared by plotting *E* for known solutions similarly treated. This curve should be renewed for each fresh batch of reagent and should be checked every 2 weeks.

Determination of CC. *CC* is determined by a somewhat modified procedure. 1 ml. of reagent 1 and 1 ml. of reagent 2 are added to 10 ml. of the test solution. The mixture is incubated for 2 hr. at 37°, cooled in ice-water for 5 min., and shaken with 5 ml. of cold ether. Photometry is carried out within 1 hr. (filter S 50 and 0·5 cm. cuvette).

Results. The colour formed with *CX* is dirty red in the water phase, but clear yellow in the ethereal solution. With *CC* the colour is red in water and in ether. Typical standard curves are given in Fig. 1. Within a wide range the relation between *E* and the concentration of the halogenated phenol is nearly linear. Concentrations of *CX* or *CC* greater than 0·5 mg./100 ml. were determined with an error of 5%. Lower concentrations may be determined

by concentrating *CX* or *CC* by extraction from acid solution with a one-third volume of toluene and transferring to a suitable volume of 0·1 N NaOH, which is then neutralized with HNO_3. The recovery is practically quantitative. The presence of 0·1 N $NaNO_3$ does not affect the test.

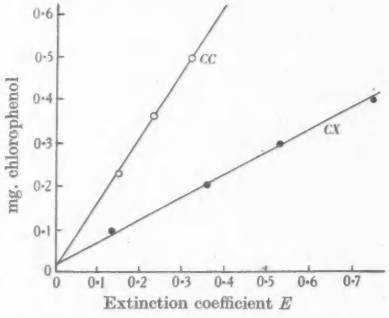

Fig. 1. Dependence of the amount of colour developed with Millon reagent on the quantity of chlorophenol employed. *CC* = *p*-chloro-*m*-cresol; *CX* = *p*-chloro-*m*-xylenol.

Discussion of procedure. By transference from the water phase into ether the coloured substance is concentrated, and at the same time clear solutions suitable for colorimetry are obtained. With *CX* the transfer is quantitative. In the case of *CC* a small fraction is retained in the water phase, but since the distribution ratio between water and ether is constant within the reported range, the value of *E* in ether remains proportional to the *CC* concentration of the original solution (Fig. 1). Furthermore, ether extraction renders the determination relatively specific, the red colour formed by phenol with the reagent being very largely ether-insoluble (see also Table 2).

The inclusion of reagent 1 in the reaction mixtures establishes the desired acidity and accelerates the colour reactions. With solutions containing substances precipitable by Millon solution, reagent 1 also serves to remove the interfering substances. In this case the test solution is centrifuged or filtered 1 min. after addition of reagent 1, and reagent 2 is then added to a portion of the cleared solution (see method for urine). Large variations in the concentration of reagent 1 (one-half to twice the indicated amount) are without effect on the intensity of the colour developed with *CX*. Therefore, the partial removal of reagent 1 by precipitation does not perceptibly affect the colour intensity obtained. In pure aqueous solutions, use of reagent 1 is, of course, not strictly essential.

Under the conditions specified, the indicated reaction times suffice to allow the colour intensity to reach a peak. Longer incubation periods are inconvenient and may entail some fading of the colour. Attempts to hasten the reaction by the use of higher temperatures were unavailing. At 60° the values of *E* were relatively low; at 95° *CX* failed altogether to give a colour with Millon reagent. On the other hand, non-halogenated phenols, as is well known, react most strongly with the reagent when boiled. Use might be made of this differential character of the reaction to determine non-halogenated in the presence of halogenated phenols. Increase in the relative amount of reagent 1 employed considerably increases the amount of water-soluble coloured substance formed with the Millon reagent by non-halogenated phenol.

B. *Procedure with biological materials*

Proteins, fats, and some inorganic salts give a precipitate with Millon reagent and interfere with its use in colorimetry. However, quantitative separation of halogenated phenols from various interfering substances present in biological material can be effected by extraction with toluene, steam distillation, and precipitation with reagent 1. The use of the two latter procedures is illustrated below.

Determination of CX in urine. 2 ml. of urine are diluted with 4 ml. of water and then 2 ml. of reagent 1 are added. After 3 min. the mixture is centrifuged, 4 ml. of the clear supernatant fluid are transferred to a test-tube, and 0·3 ml. of reagent 2 added. The solution is allowed to stand for 30 min. at room temperature, cooled, extracted with 4 ml. of chilled ether, and colorimetry carried out as in the method for aqueous solutions. A standard curve is constructed from results obtained with water solutions of known *CX* content similarly treated. The sensitive range is that covered in the experiment described in Table 1.

Determination of CC in urine. 5 ml. of urine are made just acid to litmus, transferred to a long-necked distillation flask, and steam-distilled until 15 ml. of distillate have collected. Excessive foaming is prevented by the addition of a few drops of liquid paraffin. The distillate obtained is treated as in the procedure given for determination in water. A standard curve is prepared from results obtained with aqueous solutions of known content distilled in the same apparatus and treated similarly. The sensitive range is shown in Table 1.

Determination of CX in blood. 5 ml. of oxalated blood are deproteinized by mixing with 20 ml. acetone, filtered under suction, and the precipitate washed with 10 ml. acetone and 5 ml. ether. The pooled filtrates are quantitatively transferred to a long-necked distillation flask, joined to a water condenser and held over a water-bath. 0·3 ml. 40% NaOH and, to diminish foaming, a few drops of liquid paraffin are added. The acetone and ether are then driven off slowly by heating the water-bath. Evaporation is discontinued when, the constant flow of distillate having ceased, foaming becomes excessive. The residue of liquid is then rendered acid by addition of 1 ml. 25% (v/v) H_2SO_4, and steam-distilled. When 15 ml. of distillate have collected, the distillation is stopped. The distillate or a suitably diluted portion is used as in the method for

aqueous solutions. A standard curve is prepared by subjecting aqueous solutions of known concentration to similar treatment, using the same apparatus. The sensitive range is shown in Table 1.

The same procedure can be applied to the determination of *CC* in blood.

Determination of CX and CC in minced tissues. Extraction of *CX* and *CC* is carried out from minced tissue by boiling in acetone under a reflux condenser for 1 hr. Otherwise the procedure follows the method for blood.

distillation procedures is therefore a small and, within the error of estimation, a constant fraction of the total amount originally present. Hence the incomplete recovery on distillation does not invalidate the procedure. It is therefore important, however, to standardize the distillation procedure very accurately.

Specificity of estimation. The method is highly specific for halogenated phenols. The examination

Table 1. *Recovery of halogenated phenols from biological materials*

Test substance	Medium		mg./100 ml.						
p-Chloro-*m*-xylenol (*CX*)	Urine	Added	0	7	11	24	38	57	80
		Found	0	8	11	25	36	57	78
p-Chloro-*m*-xylenol (*CX*)	Blood	Added	0	2·0	4·0	4·0	6·0	6·0	—
		Found	0	2·0	3·8	4·0	5·6	5·7	—
p-Chloro-*m*-cresol (*CC*)	Urine	Added	0	5	10	15	20	25	—
		Found	0	5	11	15	20	25	—

Results. The analytical findings when halogenated phenols were added in known amount to urine and blood are seen from representative experiments summarized in Table 1. Within the range of the reported experiments the method can be relied upon to give results with a maximum error of about 5 %.

Table 2. *Influence of phenol on estimation of p-chloro-m-xylenol (CX)*

Composition of test mixture		Analytical finding	
CX (mg./100 ml.)	Phenol (mg./100 ml.)	*E*	Estimated *CX* (mg./100 ml.)
	(*a*) Solution in water		
1·6	0	0·40	1·6
1·6	4	0·40	1·6
1·6	7	0·41	1·6
1·6	14	0·45	1·8
1·6	28	0·61	2·4
3·2	0	0·84	3·2
3·2	16	0·82	3·1
3·2	32	0·86	3·3
3·2	49	1·14	4·3
0	28	0·10	0·4
0	56	0·20	0·8
0	160	0·70	2·7
	(*b*) Solution in urine		
20	0	0·35	20
20	20	0·35	20
20	40	0·36	21
20	100	0·39	22
20	200	0·41	23
20	400	0·53	30

The absolute recovery of chlorophenol in the methods involving separation by distillation was 93–98 % in these and similar experiments on *CX*, and 83–87 % for *CC*. The loss of chlorophenol in the

of 1000 urine samples from human subjects, none of whom had received chlorophenol prior to the test, failed to reveal a single urine showing a positive reaction for halogenated phenol. The blood of normal untreated humans is likewise consistently negative.

Tyrosine does not react with Millon reagent under the conditions of the test, nor do phenol and cresol in the concentrations present in urine. The degree of interference with the estimation of *CX* resulting from the presence of unhalogenated phenol is indicated in Table 2. The relative yields of the ether-soluble coloured substance, with *CX* and phenol respectively, are at least 50 : 1. Interference with *CX* estimation was marked only when the concentration of phenol was over 10 times that of *CX*. In the presence of *CX* there is a measurable increase in the intensity of the colour of the ether-soluble material formed with phenol. The effect is, however, small and is not a serious source of error. In the method used with urine, phenol interferes with the determination of 20 mg./100 ml. only at concentrations greater than 100 mg./100 ml. (Table 2 (*b*)). The normal physiological level of 'free phenol' in urine, however, is only 5–30 mg./100 ml.

SUMMARY

1. The application of the Millon reaction to the determination of halogenated phenols in the presence of phenol is described.

2. Semi-micro-procedures for the determination of *p*-chloro-*m*-xylenol and *p*-chloro-*m*-cresol in water, urine, blood, and minced tissue are given in detail.

We are indebted to the Teva Middle East Pharmaceutical and Chemical Works Co., Ltd., Jerusalem, for a supply of *p*-chloroxylenol.

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Fate of Halogenated Phenols in the Organism

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Halogenated phenols, long employed for external disinfection, are also powerful internal disinfectants [Zondek, 1942*a*]. As the result of parenteral or percutaneous application in man, halogenated phenols are excreted in the urine, and prevent its bacterial decomposition on storage. In the clinical treatment of pyelitis and cystitis, their application may effect sterilization of the urine [Zondek, 1942*b*]. Blood from treated patients is bacteriostatic and inhibits growth of inoculated staphylococci. Successful treatment of septic infections in man is thus made possible.

The present paper is concerned with the fate in the body of *p*-chloro-*m*-xylenol (4-chloro-3:5-dimethylphenol, referred to briefly as *CX*), *p*-chloro-*m*-cresol (4-chloro-3-methylphenol, referred to as *CC*), and with the following derivatives of *p*-chloro-xylenol: acetate, benzoate, sulphate, methyl and ethyl ethers, and the *o*-iso-propyl derivative.

METHODS

The method of determining *CX* and *CC* in the presence of phenol, described by Zondek, Shapiro & Hestrin [1943], was used. *CX* and *CC* react with Millon reagent at room temperature, yielding coloured substances which can be extracted with ether and quantitatively determined therein. Phenol in the quantities present in body fluids gives no colour with Millon reagent under the conditions of these assays.

Glucuronic acid in urine was estimated according to Mozolowski [1940]. The quantity of sulphuric esters in urine was calculated by subtracting the free sulphate from the total sulphate (sulphate after acid hydrolysis).

RESULTS

Excretion of CX (p-chloro-m-xylenol) and CC (p-chloro-m-cresol) in the urine

In rabbits. Rabbits (approx. 1 kg.) were injected subcutaneously with 1 g. of *CX* dissolved in 10 ml. of olive oil. The urine was collected daily and its content of free *CX* determined. The pH was usually 7·5–8·2. About 15 % of the *CX* given was excreted during 4–5 days (Table 1). In an analogous experiment with *CC*, 15–20 % was recovered in the urine.

In man. *CX* administered intramuscularly to man is not usually excreted in the free form if the pH of the urine is acid. The difference between human beings and rabbits in respect of *CX* excretion was associated with the more acid reaction of human urine (pH 5·5–6·7). A *CX* content in urine as high as 50 mg./100 ml. and recoveries up to 8·3 % were obtained in man when the urine was made alkaline by administration of $NaHCO_3$. Concentrations of *CX* of 30 mg./100 ml. convert urine into a bactericidal fluid. If the urine is again rendered acid by administration of NH_4Cl, *CX* excretion ceases (see Table 2). But in spite of the fact that no *CX* could be demonstrated chemically, some bactericidal substance seems to have been excreted, since with most treated patients no ammoniacal

Table 1. *Excretion of p-chloro-m-xylenol in urine of rabbits*

Days after injection	Excretion of free substance by rabbit no. 1 (mg.)	2 (mg.)	3 (mg.)	4 (mg.)
1	43	40	10	30
2	46	60	50	28
3	50	30	44	32
4	25	10	34	30
5	12	—	6	8
6	—	—	—	—
Total excreted	176	140	144	128

Average excretion = 150 mg. = 15 % of quantity injected.

Table 2. *Effect of urinary* pH *on the excretion of p-chloro-m-xylenol in man*

Patient D.H.

Date	Material injected (g.)	Additional treatment	pH of urine	Material excreted (mg.)
17–18 Jan.	7	None	—	—
19–21	12	$NaHCO_3$	7·6	1320
22	0	,,	7·2	980
23	0	,,	7·1	300
24–26	0	NH_4Cl	5·2–5·8	60
27–28	8	,,	5·4	—
29	6	$NaHCO_3$	5·4	—
30	6	,,	7·2	240
31	0	,,	7·8	330
2 Feb.	3	,,	7·8	170
3	0	None	6·4	60
4	0	None	6·2	—
5	0	None	6·3	—
Total given	42	Total excreted = 3460 mg. = 8·3 %		

decomposition of the stored urine occurred. In other experiments decomposition of urine was found to be prevented by concentrations of *CX* exceeding 2 mg./100 ml.

When *CC* was administered to several human patients intramuscularly in oil, it was in no case recovered in the urine in appreciable quantity. It was extremely difficult to make the urine of these patients alkaline, an effect which was apparently due to the administration of *CC*. Where alkalinization was effected a negligible excretion of *CC* was observed.

Combined forms of CX in urine

Since phenol appears in urine combined with glucuronic acid and with sulphuric acid as well as in the free state, it was to be expected that *CX* too would be found partly combined in these forms. The combined *CX* was determined by estimating glucuronic acid and bound sulphuric acid before and after *CX* treatment. In humans injected with 5 g. *CX*, approximately 700 mg./100 ml. were excreted combined with glucuronic acid, and 840 mg./100 ml. as a sulphuric ester; 14 % of the quantity injected is thus found in the form of glucuronide, and 17 % as sulphuric ester. The total recovery of *CX* in combined form is thus 31 %.

Dissociation of combined CX by urine

Some urine samples, which give no chemical tests for free *CX* when fresh, may develop considerable quantities on standing. This finding was corroborated by bacteriological tests. Urine which contained *Escherichia coli* when fresh, became sterile after standing for some days, and in some cases then also destroyed added bacteria.

Urine specimens in which no free *CX* could be demonstrated were stored for 1 month. Control specimens were boiled before storing. Free *CX* was then determined, and 9–17 times as much was found in the unboiled urine as in the boiled specimens. These results suggest that the dissociating agent is destroyed by heat and is of an enzymic nature, presumably a glucuronidase or sulphatase. This point is under investigation.

Excretion of CX in urine after injection of CX esters and ethers

Ester derivatives of hormones have been of great value in hormone therapy (e.g. oestradiol benzoate, testosterone propionate, desoxycorticosterone acetate, etc.). The high activity of such esters is attributable to their slow absorption on subcutaneous administration and the consequent sustained liberation of active substance in the body. It seemed possible that in phenol-chemotherapy, too, the use of ester derivatives would be advantageous. The acetic acid and benzoic acid esters of *CX* were less toxic in animals and more soluble in oil than was *CX* itself (solubilities—20 and 25 % respectively for the esters, as against 10 % for *CX*). These ester derivatives could therefore be safely administered in larger single doses. It was found, also, that *CX* dissolves in oil to a higher concentration in the presence of *CX*-esters. In oil containing a total of 30 % of *CX*-acetate, for instance, 20 % of *CX* could be dissolved as against only 10 % in pure oil. The sulphuric acid ester presented a particular advantage, its sodium salt being soluble in water up to 5 %. *CX*-methyl- and ethyl-ethers were liquids at 30° and could be injected without dilution.

It was thus of interest to ascertain whether esters and ethers of *CX* are decomposed in the body with regeneration of active *CX*. The excretion of free *CX* in rabbit urine after injections of the ester serves as an indication of such regeneration. The *CX*-esters and ethers do not give a positive colour reaction with Millon reagent.

CX-acetate and benzoate are readily absorbed and decomposed to *CX* in the animal body (Fig. 1).

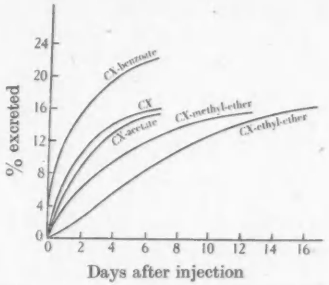

Fig. 1. Total amount of *p*-chloro-*m*-xylenol (*CX*) excretion in rabbits, as % of amount injected.

The total amount of free *CX* excreted in urine in such cases is quite as high as when *CX* itself is injected, though the rate of excretion is somewhat slower.

CX-methyl- and ethyl-ethers are also decomposed to give free *CX*, the extent of the excretion of *CX* being equal to that obtained when *CX* itself was injected. This is of particular interest, since it is not generally accepted that the animal organism can perform such dealkylations. Excretion of free *CX*, after the ethers had been given, continued for 11–17 days, as against a period of excretion of 4–6 days when free *CX* or *CC*, and esters of *CX*, were tested. Repetition of the experiment with various modifications consistently confirmed this finding. The slow absorption or decomposition of the test substance should be of clinical importance.

After injection of *CX*-sulphate no free *CX*, or only small amounts, appeared in the urine. *CX*-sulphate, which is a detoxication product of *CX*, is

presumably rapidly excreted, largely in an unchanged condition. After *o*-iso-propyl-*CX* had been given to human beings or to rabbits it was not found in the urine. Its water solubility is low and it is also practically insoluble in dilute alkali.

Concentration of CX in blood

Concentrations in blood of 3–10 mg./100 ml. are observed only when large amounts of *CX* have been given, e.g. 50 g. to man or 1–3 g. to a rabbit. These large doses can be administered by inunction of a 30% *CX*-ointment. Concentrations in blood of 1 mg./100 ml. were obtained when 1 g. of *CX* was given to man by intramuscular injection. The maximal *CX* content was observed 1–5 hr. after treatment, but in most cases no *CX* was chemically demonstrable in the blood. Nevertheless, we have sufficient evidence from the urinary data of the completeness of the absorption of *CX*. Evidence was also provided by the *CX* content of cantharidin blisters in *CX*-treated patients. Despite our inability to detect *CX* in the blood by chemical means, we have found that the blood of the treated patients showed considerable bacteriostatic power towards inoculated cocci. Whereas chemically detectable quantities of *CX* disappear from the blood several hours after treatment, bacteriostasis sets in only after more than 24 hr. The cause of this disparity will be dealt with elsewhere.

CX in cerebrospinal fluid

In no instance were chemically detectable quantities of *CX* present in cerebrospinal fluid taken from rabbits or from men at various intervals 4–48 hr. after *CX* treatment.

Fate of CX injected in the body

As excretion in the urine of free and combined *CX* accounted for only part of the *CX* injected, it was of importance to establish what proportions of the amount administered are excreted in the faeces and retained in the body. Five rats (70–80 g.) were given 100–200 mg. of *CX* by injection, and the urine and faeces collected for 48 hr. The animals were then killed, minced and extracted first with cold and then with warm acetone, and *CX* determined in the extracts. About 15% of the *CX* injected was recovered in the faeces. Half an hour after injection, 85–90% of the *CX* was still present in an unchanged form in the whole body. After 48 hr. only 10% could be recovered from the minced carcass. The total quantities of *CX* found in urine (combined + free = approx. 30%), faeces (15%), and in the body (10%) after 48 hr. thus accounted for only approx. 55% of the quantity injected. It must be assumed that some part of *CX* injected is changed by the body to a substance which cannot be detected by our procedure for the estimation of *CX* in tissues.

Absorption of injected CX and its distribution in the body

200 mg. of *CX* in 2 ml. olive oil were injected subcutaneously into the left forelegs of five rats weighing 100–120 g. After 24–48 hr. the animals were killed. The posterior and anterior parts of each were minced and analyzed separately for *CX*. The bulk of the recovered *CX* was found in all the animals in the anterior part of the body where the injection had been given. In one typical experiment, 24 hr. after the administration, 60% of the injected quantity was found in the anterior part and only 5% in the posterior. After 48 hr. the anterior part contained 6% and the posterior 1% of the quantity injected. Absorption of *CX* is therefore a comparatively slow process.

SUMMARY

1. When *p*-chloro-*m*-xylenol (*CX*) is injected into rabbits, approximately 15% of the amount administered is excreted in urine. In human urine of acid reaction only very small quantities (about 2 mg./100 ml.) are excreted. These quantities are usually sufficient to inhibit ammoniacal fermentation of the urine. When human urine is rendered alkaline by administration of $NaHCO_3$, *CX* concentrations up to 50 mg./100 ml. are obtained, and up to 8% of the administered *CX* is excreted. Concentrations of *CX* above 30 mg./100 ml. render the urine bactericidal.

2. After administration to human beings or to rabbits *o*-iso-propyl-*CX* is not excreted in the urine.

3. *p*-Chloro-*m*-cresol injected into rabbits is rapidly excreted in the urine. In man no excretion was observed.

4. The acetic acid and benzoic acid esters and the methyl- and ethyl-ethers of *CX* decompose in the body with regeneration of *CX*, which is excreted in the free form in the urine. The absorption of the esters is somewhat slower than that of *CX* itself. The time required for complete absorption and excretion of *CX*-ethers is three times that for *CX* itself.

5. *CX* is partly (30%) excreted in the urine as glucuronide and sulphate ester (in equal amounts) after administration to man.

6. Even when large doses are administered the concentration of *CX* in blood seldom exceeds 1 mg./100 ml. Concentrations of 3–10 mg. are encountered occasionally, but only when very large amounts are given. The maximum quantities in blood are found 1–5 hr. after treatment.

7. No *CX* was detected in cerebrospinal fluid after administration of this substance.

8. The aggregate amount of *CX* found in the urine and faeces and recovered from the animal body after 48 hr. accounted for only approximately 55 % of the amount of *CX* given.

9. The absorption of injected *CX* is a slow process and is incomplete even 48 hr. after the injection.

We are indebted to the Teva Middle East Pharmaceutical and Chemical Works Co., Ltd., Jerusalem, for a generous supply of *p*-chloroxylenol.

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The Replenishment of Depleted Skeletal Reserves of Magnesium

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It has been shown that the Mg of the skeleton is mobilized in response to the threatened shortage of the element in the soft tissues that results from dietary insufficiency of Mg [Duckworth, Godden & Warnock, 1940]. In the face of the persistent demand for Mg in Mg deficiency it was found that the rate of skeletal growth determined the amount that could be liberated on account of the demand of the skeleton itself for Mg [Duckworth & Godden, 1941]. The most striking feature of the mobilization of Mg was the high speed with which large quantities were released.

The question that immediately suggests itself is whether the skeletal stores can be replenished as easily as they are depleted. The problem is of practical interest in the nutrition of farm animals and man, in which the dietary intake of Mg is liable to vary. This is especially true in the case of man for whom vegetables, the supply of which fluctuates throughout the year, are the most important single source of Mg [Duckworth & Warnock, 1942–3]. The result is that varying degrees of inadequacy or alternating periods of adequacy and inadequacy are more common than constant minor degrees of insufficiency. This state may be further accentuated by losses in sweat, which may become abnormal owing to seasonal change in temperature or conditions and intensity of work [Duckworth & Warnock, 1942–3].

The present study was undertaken to examine this problem, and because of the profound influence of the rate of bone growth on liberation of skeletal Mg, a combination of Mg deficiency with different degrees of Ca deficiency was used. This approach also allowed the examination of certain points of interest concerning the interrelation of Ca and Mg in the skeleton.

METHODS

Diets used. The preparation of the diet, the methods of analysis and the management of the animals have been reported [Duckworth *et al.* 1940; Duckworth & Godden, 1941] and need not be repeated here. The technique for preparing a diet low in Mg simultaneously removes the Ca with about the same degree of efficiency, so that no additional procedure is required. The diets contained less than 6 parts per million (p.p.m.) of Mg and about 1–2 p.p.m. of Ca.

Four diets were prepared, each with a P content of 0·35 %. By additions of $CaCO_3$, with slight compensatory adjustments in the starch content, these diets were arranged to contain 0·90, 0·30, 0·10 and 0·00 % of Ca, and are referred to as diets A, B, C and D respectively.

Animal management. One litter of eight rats (4 ♂ and 4 ♀) at weaning was allotted to each diet. Of each litter six rats were placed on the appropriate deficient diet while two remaining rats of the litter were given the same diet supplemented with sufficient $MgSO_4$ to raise the Mg content to 0·07 %. The six animals undergoing skeletal Mg depletion were observed closely, and upon the death of one member of the set another, judged clinically to be close to death, was dosed with several drops of a solution of $MgSO_4$ and then transferred to the same diet supplemented with Mg. Thus of each litter three animals (hereafter referred to as 'depletion rats') died of Mg deficiency and three others (subsequently referred to as 'curative rats') were sacrificed after each had received a curative period of 10 days on the appropriate Mg-supplemented diet. The two remaining animals were treated as controls, one being killed at what was estimated to be the average

time of survival of the three depletion rats and the other being killed 10 days later. These animals are referred to as the 'depletion control' and the 'curative control' respectively.

The animals were under constant supervision during the day and were visited every 4 hr. during the night. Thus differences between the corresponding depletion and curative animals, from the standpoint of length of time on the Mg deficient diets, were minimized.

RESULTS

The average values for bone composition are given in Table 1. Individual values for the percentage of Mg in bone ash are given in Fig. 1.

Table 1. *Composition of bones of rats receiving different types of diets*

Group*	Wt. of bones (mg.)	Wt. of ash in bones (mg.)	Wt. of ash in bones (%)	Wt. of Mg in ash (mg.)	Wt. of Mg in ash (%)	Mg deposited during curative period (mg.)
A —	362	204	56·4	1·14	0·56	
A — +	524	312	59·5	2·46	0·79	1·32
A 1 +	350	201	57·4	1·94	0·97	
A 2 +	544	332	61·0	3·00	0·90	
B —	363	195	53·7	1·10	0·57	
B — +	565	327	57·9	2·51	0·77	1·41
B 1 +	370	207	55·9	2·00	0·97	
B 2 +	642	374	58·3	3·19	0·85	
C —	388	191	49·2	1·06	0·56	
C — +	461	235	51·0	1·95	0·83	0·89
C 1 +	406	208	51·2	2·08	1·00	
C 2 +	448	227	50·7	2·12	0·93	
D —	279	135	48·4	0·76	0·56	
D — +	266	116	43·6	1·08	0·93	0·32
D 1 +	268	122	45·5	1·31	1·07	
D 2 +	320	130	40·6	1·42	1·10	

* — indicates depletion rats; — + indicates curative rats; 1 + indicates depletion control rats; 2 + indicates curative control rats. Letters indicate the diet used (see 'Methods').

The average survival periods of the depletion rats on Mg-deficient diets A, B, C and D were, respectively, 5·0, 7·3, 8·7 and 11·3 days. Comparison of the data for bone growth should be made with these differences in mind. Since a more extensive study has already been published [Duckworth & Godden, 1941] of bone development in rats receiving the present diets, further discussion is unnecessary.

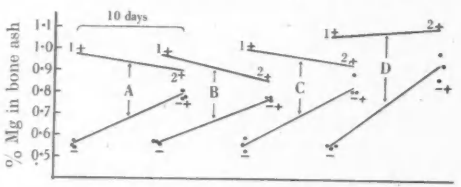

Fig. 1. Percentages of Mg in the bone ash of rats receiving different diets and treatment. — indicates depletion rats; — + indicates curative rats; 1 + indicates depletion control rats; 2 + indicates curative control rats. Letters indicate the diet used (see 'Methods').

The percentage of Mg in the ash was about the same in all depletion rats irrespective of diet. After 10 days of re-alimentation with Mg the influence of different degrees of Ca adequacy became apparent. Thus the curative rats receiving diets A and B showed closely similar percentages of Mg in the ash, but the curative rats receiving diets C and D showed progressively higher concentrations with decreasing Ca intake. It will be noted that the individual values were not so closely spaced in the two latter groups. The control rats receiving diets A, B and C showed the characteristic reduction of Mg percentage with increasing age. The control rats receiving diet D did not show this reduction and the values were above those of controls receiving the normal diets A and B. There is, perhaps, some indication of increased Mg percentage in the control rats receiving diet C. In none of the curative animals was the value of the corresponding curative control reached, although one rat on diet C and all on diet D approached, or surpassed, the curative control values for diets A and B.

The amounts of Mg in the bone were closely similar for corresponding rats of diets A and B. The curative rats and the curative control of diet C showed, by the lower quantities of Mg and ash, the influence of restricted Ca intake that had not been indicated in the depletion rats and the depletion control, which had received the diet for a shorter period. The amounts of Mg in the bones of rats receiving diet D were lower in all cases than for corresponding rats on other diets.

The quantities of Mg stored by the curative rats, estimated by difference between the values for depletion and curative animals, were about the same in rats of diets A and B. The value was less in animals receiving diet C and least in those receiving diet D.

The symptomatology, including cessation in growth, did not differ from that of previous studies. After the addition of Mg, curative rats of diets A and B grew normally, rats of diet C showed subnormal growth and those of diet D slight increases.

DISCUSSION

The regularity of the individual values for the percentage of Mg in bone ash, given in Fig. 1, indicates that the differences observed in the curative littermates at the time they were killed can be attributed to differences in the degree to which Mg is stored under different dietary conditions. This is supported

by the close similarity in the results observed for the curative rats receiving diets A and B, which are essentially normal diets.

It is well known, and the evidence has been summarized elsewhere [Duckworth, 1938–9], that when demineralization of the skeleton occurs the Mg content of the bone ash generally increases. Because of the proportional increase in organic matter, itself containing Mg, during demineralization, it has not been possible to state categorically that the increase found in the percentage of Mg in the bone ash arose from an increase in the Mg content of the crystals of bone salt. The possibility that the increase was the outcome of a greater amount of organic matrix remained.

The present findings contribute information about this aspect of the interrelation of Ca and Mg in bone. The Mg content of the ash of the control animals increased as the Ca supply diminished, the customary reduction with age giving way to an increase with progressive demineralization in the case of diet D. In the depletion rats this did not happen, irrespective of the degree of calcification of the bones. The curative rats receiving diets C and D showed progressively greater percentages of Mg in the ash as Ca intake fell. The simplest explanation of these changes is that Mg can replace Ca, to a limited extent, in the bone salt crystal during periods of Ca insufficiency. If, however, there is simultaneously a deficiency of both Ca and Mg, then this replacement does not occur. When, after a period of both Ca and Mg lack, the Mg is restored to the diet, it then begins to exert its Ca-sparing effect in the skeleton. It cannot be argued convincingly that the increase in the percentage of Mg in the ash of rats receiving diet D arose in any way from the greater percentage of organic matter. This would imply that the organic matrix of bone contained many times more Mg/unit weight than other tissues [the data are collected in the review of Schmidt & Greenberg, 1935] and that this higher content could be dispensed with in Mg deficiency to give the constant results found for the depletion rats.

Tufts & Greenberg [1937–8*a*] showed that the Mg content of soft tissues is not reduced in Mg deficiency. Thus, when Mg is restored to the diet after a period of deficiency, there is no soft tissue deficit to remedy, and the only requirements are those for the replenishment of the skeletal reserves and for new soft tissue and skeletal growth. Since Tufts & Greenberg [1937–8*b*] obtained good growth, normal gestation and parturition, and fair lactation, in rats whose diet contained only 0·005 % Mg, it is unlikely that the present diet failed to supply sufficient Mg to meet the above demands. This failure of the curative rats to attain the same percentage of Mg in the ash as the corresponding curative control rats may have arisen from either of two main causes, or from a combination of them. The first cause of failure may be that Mg cannot re-enter the depleted crystal, or that it can only re-enter it at a very slow rate. The second may be that new bone salt deposited when Mg is restored to the diet does not initially contain the normal quantity of Mg, normal composition being attained only gradually. That this failure almost certainly involves the former of these alternatives is shown by earlier studies [Duckworth & Godden, 1941], where it was found that the return of plasma Mg to normal levels during the second phase of the deficiency, when drafts on the skeletal Mg deposits have ceased, was not accompanied by a return of Mg to the skeleton. A further opportunity for Mg to enter bone salt would be during those processes of solution and redeposition of bone salt which, although detected, have yet to be measured accurately. This additional process was not considered above when the replenishment of the reserves of the growing animal was discussed, but it may have been mainly responsible for the re-entry of Mg into the bone salt, in the curative rats receiving diet D in which no bone growth occurred.

Irrespective of the manner in which Mg returns to the depleted bone salt it is clear that the reparation of the deficit is less rapid than the depletion process. Attention has been called elsewhere [Duckworth & Warnock, 1942–3] to the wide variations in the concentrations of Mg in human bone, and to the evidence of dietary deficiencies in some social groups presented. It may be that the variation noted is evidence not only of depletion but also of the slow rate at which the deficit is made good when Mg supplies become adequate. It might be expected that in the adult the return of bone composition to normal, in respect of Mg, would require longer than in the young growing animal, because of the absence of active bone growth. In this case, however, the mechanism involved may resemble that of the curative rats of diet D, and may depend on the processes of solution and re-precipitation mentioned above.

SUMMARY

1. Evidence is presented to show that the increase of Mg in bone ash which occurs in demineralization arises from a Ca-replacing action of Mg. It does not occur if Mg supplies are inadequate.

2. The replenishment of skeletal Mg reserves, in rats depleted by acute Mg deficiency, is incomplete after a 10-day period of re-alimentation with Mg, irrespective of whether or not bone growth occurs.

3. The interrelation of Ca and Mg in the skeleton is discussed in the light of the experimental findings

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The Iodometric Micro-determination of Arsenic in Biological Material

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The method of Allcroft & Green [1935; see also Kiese, 1937] for the titrimetric estimation of arsenic in tissue digests yielded satisfactory results with 0·05–1 mg. As. As is separated as AsH_3, which is passed into $AgNO_3$ solution, the arsenite formed being titrated with dilute I_2. It has been found possible to adapt this technique for 5–50 μg. As by reducing the volume of the solution to be titrated till the I_2 required for a distinct colour change is a small fraction of that required to oxidize the arsenite.

Tubes have been constructed in which 1·5 ml. N/50 $AgNO_3$ are sufficient for efficient absorption of AsH_3 at the rapid rate of passage of H_2 necessary for complete separation of As as AsH_3. Titration with N/200 I_2 is carried out in the absorption tube. Under the conditions of the experiment, a distinct colour change is produced by 0·003 ml. I_2, although the amount may vary slightly with the observer and with the lighting. The microburette described by Conway [1939], with a ground-glass joint in place of the rubber stopper in the standard model, is used in the titration. If the reservoir is shielded from light, there is little change in the I_2 over intervals of weeks.

The procedure described has been found reliable in thousands of routine estimations carried out by several workers in these departments and elsewhere. No difficulty has been encountered in adapting it to many types of material.

OUTLINE OF METHOD

Organic matter is destroyed by digestion with a mixture of HNO_3, $HClO_4$ and H_2SO_4. The digest is treated with $(NH_4)_2C_2O_4$ to remove interfering N compounds. When the biological material is rich in As and the sample taken for analysis is small ('Digestion—small scale'), AsH_3 is evolved directly from the digest. With large samples of material ('Digestion—large scale'), As is distilled from the digest as $AsCl_3$, and AsH_3 is evolved from the acid distillate. The procedure thenceforth is the same in both cases. After adjusting the acid concentration and adding $CuSO_4$ and $SnCl_2$ to catalyze H_2 evolution, Zn is introduced into the As-containing solution and the gas liberated is passed through 10N NaOH and N/50 $AgNO_3$. The dimensions of the $AgNO_3$ tube are shown in Fig. 1. To avoid risk of breaking the tip of the Conway burette, the gas inlet (thick-walled capillary tubing of 1 mm. internal diam.)

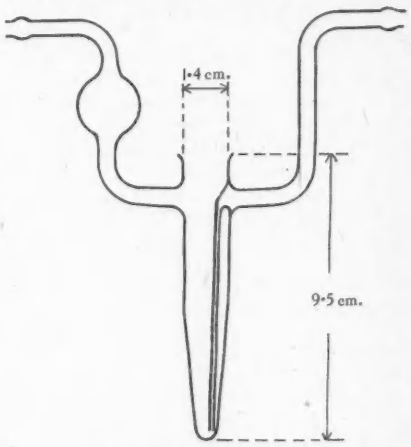

Fig. 1. The absorption tube.

should be situated close to the wall, as shown. Before titrating the arsenite in the absorption tube, solid KI is added to remove excess Ag^+ which would otherwise prevent the appearance of the I_2-starch colour. This is followed by $NaHCO_3$ to remove HI produced in the titration, and starch indicator. The tip of the Conway burette is immersed in the solution, which is stirred during the titration by passing air through the gas inlet of the absorption tube.

Since a large number of digestions can be carried out simultaneously, the time involved per sample at this stage of the determination is negligible. By working in serial fashion with six sets of apparatus, estimations can be put through the stage of AsH_3 separation and arsenite titration at about 10 to the hour. When preliminary separation of As as $AsCl_3$ is necessary, the distillations can be done at the rate of one every 10 min. by using duplicate apparatus.

EXPERIMENTAL

Reagents

All reagents should be of analytical or As-test quality

HNO_3, conc.	$SnCl_2$, 40% in conc. HCl
$HClO_4$, 60% (w/w)	$CuSO_4$, $5H_2O$, 5%
H_2SO_4, conc.	Zn shot, freed from dust and extra large shot
HNO_3, fuming	H_2SO_4, 20% (w/v)
$(NH_4)_2C_2O_4$, sat. solution	KI, cryst.
$FeSO_4$, $7H_2O$, cryst.	$NaHCO_3$, powdered
KBr, cryst.	Starch solution, fresh
NaCl, cryst.	I_2, N/200
$AgNO_3$, N/50	Standard arsenite solution, prepared from As_2O_3
NaOH, 10N	
Glycerine	

Procedure

Digestion—small scale. Up to 5 ml. blood, 10 ml. urine or 2 g. tissue are measured into a 100 ml. Kjeldahl flask, and conc. HNO_3 (5 ml.), $HClO_4$ (3 ml.), conc. H_2SO_4 (10 ml.) and two or three clean glass beads are added. The flask is warmed, with shaking, over a naked flame till brown fumes are given off, and heating is continued on a digestion stand over a very small flame (to avoid bumping) till they are no longer evolved. The contents are then gently boiled till H_2SO_4 fumes appear, and the clear liquid becomes colourless. Heating is continued for 10 min. If the mixture chars at any time, the flask is cooled slightly and a few drops of fuming HNO_3 added.

When digestion is complete, the flask is allowed to cool and $(NH_4)_2C_2O_4$ solution (5 ml.) is added. The liquid is brought to the boil and heating continued till white fumes appear. Distilled water (5 ml.) is added to the cooled liquid, which is again brought to the boil, and heating continued till 5 min. after the first appearance of white fumes.

Control experiments, with reagents alone, are carried out in exactly the same manner. It is important that heating of the digest at each stage should be continued for at least the period specified.

Digestion—large scale. Tissue. Up to 20 g. of minced, moist tissue are wrapped in very thin paper and placed in a 300 ml. Kjeldahl flask (with ground joint). After the addition of conc. HNO_3 (30 ml.) and two or three clean glass beads, the flask is set aside overnight.

Blood. Up to 30 ml. blood are treated as described above.

Urine. To not more than 150 ml. urine one-fifth its volume of fuming HNO_3 and two or three glass beads are added, the liquid evaporated to about 10 ml., and the digestion then carried out.

$HClO_4$ (15 ml.) and conc. H_2SO_4 (20 ml.) are added to the flask, which is warmed cautiously with shaking over a naked flame till reaction sets in, and is then placed on a digestion stand. After the first violent reaction subsides, the burner is lit and the contents brought to the boil. When digestion is complete, 20 ml. of $(NH_4)_2C_2O_4$ solution and of water are added. It may be necessary to make several additions of fuming HNO_3 before digestion is complete.

$AsCl_3$ *distillation.* This is necessary only after large-scale digestion. A delivery tube is connected, with conc. H_2SO_4 as lubricant for the ground-glass joint, to a 150 ml. Fresenius flask containing distilled water (40 ml.) immersed in ice-water [see Bang, 1925].

Distilled water (50 ml.) is added to the Kjeldahl flask and the contents are cooled thoroughly. Through a wide-bore funnel, $FeSO_4$, $7H_2O$ (8 g.), KBr (2 g.) and NaCl (25 g.) are added and the flask immediately connected to the previously lubricated joint of the delivery tube. A 200 ml. volumetric flask is placed over the exit of the Fresenius flask. The liquid in the Kjeldahl flask is boiled vigorously for exactly 10 min., the ice around the receiver being renewed as necessary. With the liquid still boiling, the delivery tube is disconnected from the Kjeldahl flask. The contents of the Fresenius flask are then transferred to the 200 ml. volumetric flask, and, when cool, diluted to the mark.

Separation of As *as* AsH_3. Two absorption tubes (Fig. 1), the second of which acts as guard tube, are charged with N/50 $AgNO_3$ (1·5 ml.). The tubes are stoppered and connected to a small wash-tube containing 10N NaOH (renewed after 12 estimations), glycerine being used as a lubricant for rubber tubing. A right-angle tube, bearing a stopper to fit the AsH_3 generation flask, is attached to the wash-tube.

After a small-scale digestion, the contents of the Kjeldahl flask are transferred to a 150 ml. conical flask, with 40 ml. distilled water as washing fluid. Alternatively, a measured fraction of the diluted digest is made up to 50 ml. with 20% H_2SO_4. 40% $SnCl_2$ (10 drops) and 5% $CuSO_4$ (3 drops) are added. The liquid is warmed if necessary, Zn shot (15 g.) quickly introduced, and the flask at once connected to the previously prepared absorption apparatus.

After a large-scale digestion, if the portion taken is less than 100 ml., it is made up to this volume in a 150 ml. conical flask with 20% H_2SO_4. When the total distillate is used, it is transferred to a 250 ml. conical flask. 40% $SnCl_2$ (10 drops/100 ml. total vol.) and 5% $CuSO_4$ (3 drops/100 ml. total vol.) are added and the liquid is warmed. Zn (25 g.) is introduced and the flask immediately connected to the absorption apparatus.

Titration of arsenite. When H_2 has been passed through the absorption apparatus for 30 min. the tubes are disconnected. The rubber stopper of the absorption tube is washed down with a few drops of distilled water, and the gas inlet is connected to the compressed air. KI is added till the precipitated AgI redissolves. After the addition of a spatula-point of $NaHCO_3$ and a drop of starch solution the tube is slipped over the jet of the Conway burette. It is held in place by sliding the titration platform under it, and with air passing through the liquid at a gentle rate I_2 is run in till a red-brown colour appears and persists for 30 sec. Before the end of the titration, the gas exit is washed down by increasing the flow of air till a little of the liquid is blown into it. A daylight lamp is of advantage in observing the end-point. If the liquid in the guard tube darkens, it is titrated in the same way.

At the beginning of each day, the glass tubing in the burette is thoroughly washed out with fresh I_2 from the reservoir. To standardize the I_2, two different quantities of arsenite solution are measured into absorption tubes, the volume made up to about 2 ml. with N/50 $AgNO_3$, and the titrations carried out as described above. The amount of As (μg.) is plotted against ml. I_2, and the analytical results are read off from the straight line joining the two points. This line cuts the I_2 axis at the volume required to cause a colour change in 2 ml. N/50 $AgNO_3$ treated as above, in absence of arsenite.

RESULTS

In 20 experiments in which 20 μg. As were added as Na_2HAsO_3 to 100 ml. 20 % H_2SO_4 and separated as AsH_3, the mean recovery was 19·6 μg., and the standard deviation of a single observation from the mean was 0·73 μg. In the subsequent pages of this paper, 'standard deviation' always means the standard deviation of a single observation from the mean. In 26 determinations of 20 μg. As added to 3 ml. blood for small-scale digestion, average recovery was 18·9 μg. (standard deviation 2·3 μg.). These two series of estimations were carried out by a technical assistant who understood that he was dealing with amounts of As differing slightly from each other.

In 19 analyses in which three portions of the same digest, averaging 12·1 μg. As, were taken for the final stage (separation of AsH_3 and titration of arsenite) of the estimations, the standard deviation was 0·50 μg. As. A research worker investigating the procedure found the average recovery in 24 determinations of 20 μg. As added to 1 ml. blood to be 18·8 μg. (standard deviation 2·5 μg.). The results in Table 1 for As added to 20 g. of minced mouse illustrate the recovery after large-scale digestion and preliminary separation of As as $AsCl_3$.

Table 1. *Recovery of arsenic added to minced mouse prior to large-scale digestion*

As added (μg.)	As recovered (μg.)	Recovery (%)	As added (μg.)	As recovered (μg.)	Recovery (%)
8·0	7·4	93	48·0	46·3	97
10·0	9·3	93	50·0	45·8	92
12·0	12·4	103	56·0	55·6	99
16·0	17·8	111	60·0	54·7	91
20·0	17·5	88	64·0	60·1	94
30·0	32·2	107	72·0	63·4	88
32·0	30·6	96	72·0	69·4	96
40·0	42·4	106	80·0	77·2	97
40·0	38·1	95	80·0	77·1	96
40·0	34·4	86	100·0	96·6	97
48·0	47·6	99			

The accuracy is largely determined by the care taken in carrying out the control experiments with reagents alone. The lower limit for accurate estimations depends upon how much As is present in the reagents, the lighting during titrations and the sensitivity of the individual to the end-point. Below 10 μg. As, the titration error (about 0·3 μg.) assumes increasing importance as the amount of As decreases. The percentage As not retained in the first $AgNO_3$ tube varies with the rate at which H_2 is developed, but as a rule little AsH_3 reaches the guard tube with less than 50 μg. As or passes through it with less than 100 μg. present. With special care it is possible to work in the range of 2–10 μg. As with the accuracy found for larger amounts of the element.

DISCUSSION

Allcroft & Green [1935] found HNO_3 and $HClO_4$ to be the most satisfactory reagents for digestion in their As determinations. Their figures, however, showed a constant loss of As, particularly marked with 5 and 10 μg. As_2O_3, which should probably be attributed to the digestion rather than to titration or separation of AsH_3. In trials carried out here, poor recoveries were invariably obtained when H_2SO_4 was omitted from the digestion mixture. With a mixture of all three acids [Kahane, 1932; Hinsberg & Kiese, 1937; Cassil, 1937], loss of As during the combustion has not been noted, even though no special precautions were taken when fairly large amounts of Cl^- were present [see How, 1938; Klein, 1920].

The Sub-Committee of the Society of Public Analysts on the Determination of As [1930], Winterfeld, Doerle & Rauch [1935] and Kiese [1937], all found it necessary to carry out a double separation of As after digestion of large samples of biological material. NaCl is employed as a source of gaseous HCl in the preliminary separation of As as $AsCl_3$. Bang [1925] recommended KCl. T. B. B. Crawford & I. D. E. Storey (private communication) found a 20 % loss of As when HCl [Cox, 1925; Ramberg, 1921; Winterfeld *et al.* 1935; Kiese, 1937] was used. With small amounts of blood or urine, Dr Ena Brown (Mrs G. M. Henderson) has dispensed with the $AsCl_3$ distillation without increasing the error. The separation of As as AsH_3 directly from acid digests was recommended by Allcroft & Green [1935].

The most satisfactory reaction with Zn, in presence of Cu^{++} and Sn^{++}, for the separation of As as AsH_3, is obtained with 4N H_2SO_4. A similar rapid, but not violent, evolution of H_2 from the $AsCl_3$ distillate is obtained after it has been diluted to 200 ml., giving a concentration of HCl of approximately 2N. The use of NaOH to remove H_2S from the stream of AsH_3 and H_2 was recommended by Hinsberg & Kiese [1937]. According to Kiese [1937], N/50 $AgNO_3$ is sufficiently dilute to avoid the formation of any arsenate in the reaction with AsH_3. Our results indicate that both the separation of As as AsH_3 and the conversion into arsenite proceed quantitatively. Contrary to the suggestion of Conway [1939], we have found air stirring to be entirely suitable for iodometric titrations, in spite of the volatility of I_2.

Methods based on the matching of stains have been considered unsatisfactory by many authors, e.g. Winterfeld *et al.* [1935], Cassil [1939]. Thus Barnes & Murray [1930] found a standard deviation of 4·8 μg. in 150 estimations of 27·3 μg. As_2O_3, and Neller [1929] a 15 % standard deviation in the recovery of As (22–94 μg. As_2O_3) by a similar method.

In these studies, the length of stain produced by AsH_3 on $HgBr_2$ paper was measured and the corresponding figure for As read from a graph obtained with standard solutions. It should be noted that variation in length of stain for a given amount of As enters into the preparation of the graph as well as into analysis of an unknown sample, thus introducing the possibility of further error in the final result. The standard deviation of a single determination of 20 μg. As by the micro-titrimetric procedure was 3·7 %. While it is as sensitive as the usual Gutzeit procedure, the titrimetric technique is much more objective and does not call for the same careful control of physical conditions in the evolution of AsH_3.

Cassil & Wichmann [1939] have reported a procedure for small quantities of As very similar in principle to that described above. As was separated as AsH_3 and passed into 1 ml. 1·6 % $HgCl_2$. I_2 was added and the excess back-titrated with arsenite, in contrast to our direct titration. The final volume of solution titrated was about 8 ml. After the absorption of AsH_3 in $HgCl_2$, one atom of arsenic was found to correspond to eight atoms of iodine. The sensitivity of the estimation must, therefore, have been identical with that observed in titrating, in a volume of 2 ml., the product of the reaction between AsH_3 and $AgNO_3$, which requires two atoms of iodine for each atom of arsenic. It seems that the sensitivity in using $HgCl_2$ could be further increased by decreasing the volumes of the reagents added before the back-titration. Cassil & Wichmann's accuracy was somewhat greater than that claimed for the present procedure.

SUMMARY

1. With a special tube for the absorption of AsH_3 in $AgNO_3$ solution and the Conway micro-burette for the titration of the arsenite formed, the iodometric estimation of As in digests of biological material was modified to cover the range 5–50 μg.

2. The recovery of 20 μg. As was 98 %, the standard deviation of a single observation from the mean being 3·7 %.

3. Methods for the digestion of As-containing material were examined, and the use of a mixture of HNO_3, $HClO_4$ and H_2SO_4 adopted.

4. Preliminary separation of As as $AsCl_3$ from the digest was found to be necessary only when large samples of material were taken for analysis.

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The Components of the Antigenic Complex of *Salmonella Typhimurium*

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Recent work on the antigenic complex of *Eberthella typhosa* (*Bact. typhosum*) has shown that the substance may be obtained in a state approaching chemical homogeneity by trichloroacetic acid or diethylene glycol extraction of the bacterial cells, and by tryptic digestion of the dried organisms. The complete antigenic complex may be broken down by mild acetic acid hydrolysis with the libera-

tion of the component polysaccharide, conjugated protein and lipid constituents. The polysaccharide, which is non-antigenic and relatively non-toxic, is responsible for the specific properties of the intact antigenic complex. It has been shown to be free from lactose, pentose and uronic acid residues, but it yields 40 % *d*-glucose, 21 % *d*-mannose and 17 % *d*-galactose on hydrolysis. The polysaccharide has a minimum molecular weight of the order of 10,000 [Freeman & Anderson, 1941; Freeman, 1942]. Henderson & Morgan [1938] have shown that the antigenic complex of the O 901 strain of *Eberth. typhosa* is obtained by diethyleneglycol extraction of the organisms, and exists as a polymolecular complex containing three main components, a specific polysaccharide, a protein and a phospholipin.

In the present paper the methods which have proved successful in the case of *Eberth. typhosa* have been applied to the investigation of the nature of the components of the antigenic complex of *Salmonella typhimurium* (*Bact. typhi-murium*).

The results have in general confirmed those already obtained with the closely related *Eberth. typhosa*, but quantitative differences, especially in the nature of the specific polysaccharides, which account for the immunological dissimilarity of the two species, have been found.

EXPERIMENTAL

The organism was an O-strain (L.S.H.T.M. Catalogue C 3/23), which was free from Vi antigen. It was grown on a synthetic medium as described by Freeman, Challinor & Wilson [1940]. The dried bacterial bodies and several batches of antigen used in this work were prepared in the Departments of Biochemistry and of Bacteriology, London School of Hygiene and Tropical Medicine; the author is greatly indebted to Professors H. Raistrick and W. W. C. Topley for the use of these starting materials. The antigenic complex was obtained by tryptic digestion [Freeman *et al.* 1940]; the dried organisms were digested in batches of 45 g. with an average yield of 6·9 % of antigen.

Purification of the antigenic complex

Before degradation, the antigenic material was fractionally precipitated from aqueous solution, (*a*) with ethanol and (*b*) with ammonium sulphate, in order to establish its homogeneity.

(*a*) *Ethanol fractionation of* Salmonella typhimurium *antigen.* 23·2 g. of antigen were dispersed in 600 ml. of water by grinding in a mortar and by mechanical shaking. The opalescent viscous solution was made 0·1 N to acetic acid and centrifuged at 3000 r.p.m. for 3 hr., giving 0·24 % of dark insoluble residue. The opalescent supernatant solution gave no appreciable precipitate when ethanol was added to a concentration of 50 % (w/w), but when the ethanol content was raised to 60 % the bulk of the antigen separated out after standing overnight, leaving a water-clear supernatant solution, which contained little or no antigenic material. The precipitate was washed with 60 % ethanol and redispersed in 1 l. of 0·1 N acetic acid. Addition of ethanol to 57 % concentration gave a small amount of precipitate (F0–60 %/0–57 %), which was removed by passage of the ethanol suspension through a de Laval Industrial Separator (F0–60 %/0–57 %; 1·25 g. = 5·4 %; N, 5·2; ash, 13·4; reducing sugar value, 58 %; limit of precipitation with antiserum, 1 in 338,000; optimum, 7500–37,500), and a colourless, opalescent supernatant solution. The ethanol content of the latter was raised to 60 % and the bulk of the antigen separated out leaving a clear supernatant solution. The main antigenic fraction, F0–60 %/57–60 %, was washed twice with absolute ethanol and twice with ether and dried *in vacuo* over P_2O_5 (19·1 g. = 82·4 %; N, 2·7; ash, 14·2; R.V. (reducing sugar value), 65 %; $[\alpha]_D^{18°}$ (in formamide, $c = 1$), $+106° \pm 2°$, limit of precipitation with antiserum 1 in 338,000; optimum, 7500). 50 hr. electrodialysis in cellophane membranes reduced the ash content of F0–60 %/57–60 % to 7·9 %; N, 2·8 %. The contents of the first and second 60 % ethanol supernatant solutions were practically devoid of antigenic material. Total recovery in fractionation was 97·6 %.

Reducing sugar value (R.V.) and specific rotation determinations on the isolated fractions were carried out as described by Freeman & Anderson [1941], and other analyses were by Dr G. Weiler.

(*b*) *Ammonium sulphate fractionation of antigen.* A further attempt to fractionate the antigenic material was made by means of precipitation from aqueous solution by increasing concentrations of ammonium sulphate. A preliminary experiment showed that the bulk of the antigen was precipitated between the levels of 23–35 % concentration of ammonium sulphate.

The ethanol fractionated antigen (F0–60 %/57–60 %), 14·9 g. in 500 ml. of water, was three times fractionated between the limits of 23 and 35 % concentration of ammonium sulphate. The isolated fractions were submitted to dialysis to remove the ammonium sulphate, first in collodion sacs, and then to electrodialysis for 60 hr. between cellophane membranes. The bulk of the antigenic material (10·03 g. = 67·5 %) was precipitated at 35 % concentration of ammonium sulphate (F 23–35 %; N, 1·72; R.V. 65; ash, 4·2 %; $[\alpha]_D^{17°}$ (in formamide, $c = 1$), $+95° \pm 2°$; limit of precipitation with antiserum 1 in 1,000,000, optimum 37,500). When the ammonium sulphate concentration was raised to 41 %, a further active fraction (F 35–41 %) was obtained (0·779 g. = 5·3 %, N, 2·5; R.V. 65; ash, 5·8 %; $[\alpha]_D^{17°}$ (in formamide, $c = 1$), $+91° \pm 9°$; limit of precipitation with antiserum 1 in 1,000,000, optimum 37,500). Total recovery in fractionation was 77 %.

Extraction of the antigenic material with organic solvents. Extraction of the antigenic complex with organic solvents [cf. Morgan & Partridge, 1940*a*] yielded only 17 mg. or 0·34 % of fatty fraction. It is therefore apparent that the lipid fraction which was obtained from the antigenic complex on hydro-

lysis in a yield of about 4%, as described later, was an essential antigenic component and not merely an adventitious impurity.

Properties of the antigenic complex

The antigenic complex of *S. typhimurium* gave opalescent aqueous solutions even when the concentration of antigen was as low as 0·4%. The bulk of the substance remained in solution after 30 min. centrifuging at 15,000 r.p.m. The antigenic complex also dissolved readily in formamide and in concentrated formic acid, giving solutions which were practically free from opalescence, but it was insoluble in glacial acetic acid and in dioxan. The bulk of the substance was insoluble in concentrated aqueous phenol solutions but, on exposure to 95% aqueous phenol, the antigenic material swelled up to form a thin translucent jelly which separated out on centrifuging. 0·4% aqueous solutions of the antigen gave no precipitates on addition of an equal volume of uranyl acetate (cf. antigen of *Eberth. typhosa* Ty 2), 10% trichloroacetic acid, salicylsulphonic acid, tannic acid solution, or Esbach's reagent. Phosphotungstic acid (2% in 5% H_2SO_4) gave an immediate flocculent precipitate. The presence of tyrosine residues was indicated by positive Millon's α-nitroso-β-naphthol and Ehrlich's diazo reactions. A weakly positive Sakaguchi reaction for arginine was obtained, but Ehrlich's diazo reaction for histidine was negative. 20 mg. of the substance in 1 ml. of water gave a weakly positive biuret reaction, but the ninhydrin reaction was negative. The substance gave a strong Molisch's reaction but did not reduce Fehling's solution.

On analysis the pure antigen gave C, 48·4; H, 7·3; N, 1·8–1·9; non-volatile ash, 4–5; volatile fatty acid on hydrolysis (calculated as acetyl groups), 6·1%; $[\alpha]_D^{17°}$, +95° ± 1° (in formamide, $c = 1$); $[\alpha]_D^{17°}$, +96° (in water, $c = 1$); reducing value (method of Hagedorn & Jensen [1923]) on hydrolysis with 2N HCl for 2 hr. in a sealed tube, 65%; organic P, 0·5; inorganic P, 0·3% (N/organic P ratio, 7). Determination of amino-N (Van Slyke) showed that 0·2% of N was present as amino-groups.

Fractionation of antigen from aqueous solution with acetone. To provide further evidence of the homogeneity of the antigenic preparation after purification by fractionation from aqueous solution, first with ethanol and then with ammonium sulphate, the substance was submitted to a further fractionation from aqueous solution with acetone.

Antigen (1·98 g.) was dissolved in 100 ml. water; the bulk of the substance (1·787 g. = 90·2%; N, 1·9; ash, 4·7; R.V. 63%; $[\alpha]_D$ (in formamide, $c = 1$), +95° ± 1°; limit of precipitation with antiserum 1 in 1,000,000; optimum, 37,500) was precipitated between acetone concentrations of 43 and 45% (by weight). On raising the acetone concentration to 52% a further fraction (F45–52%) was obtained (0·169 g. = 8·5%; N, 1·8; ash, 4·9; R.V. 65%; $[\alpha]_D$, +97° ± 1°; limit of precipitation with antiserum, 1 in 1,000,000; optimum, 37,500). 0·022 g. (1·1%) of substance was recovered from the supernatant solution.

Immunological properties of Salmonella typhimurium *antigen.* The antigen in its unfractionated state was toxic for mice, average lethal dose, 0·13–0·27 mg. [Freeman *et al.* 1940]. Mouse protection experiments have been carried out on several batches of crude antigen [cf. Raistrick & Topley, 1934; Topley, Raistrick, Wilson, Stacey, Challinor & Clark, 1937]; the material was shown to contain antigens comparable in immunological activity with that of the dried bacterial cells. It has not yet been possible to conduct mouse protection tests on the purified antigen, but this material stimulated the production of precipitins in rabbits at least as readily as the crude substance.

Formamide dissociation of the antigenic complex. Morgan & Partridge [1940*a*] have shown that the antigen of *Bact. dysenteriae* Shiga (a phospholipin-polysaccharide-conjugated protein complex) may be dissociated by the action of neutral formamide with the production of the free phospholipin and a polysaccharide-conjugated protein complex.

The antigen of *S. typhimurium* (1·13 g.) was submitted to dissociation by the method of Morgan & Partridge and yielded only 0·011 g. (0·9%) of a colourless, lipid fraction and 0·894 g. (79%) of 'formamide dissociated antigen'. The latter did not differ significantly in its analytical data (N, 1·8; ash, 5·2%; $[\alpha]_D^{15°}$, +101° ± 1°), limit of precipitation with antiserum and chemical properties, from the undissociated antigenic complex. The aqueous solutions were, however, slightly less opalescent than those of the original antigen, and on addition of NaOH to pH 8·5–10, solutions of the formamide-dissociated product became almost water-clear, whereas those of the antigenic complex were still definitely opalescent.

Acetic acid hydrolysis of the antigenic complex. The antigenic complex was split up into its polysaccharide, lipid and conjugated protein components by hydrolysis with dilute acetic acid [Freeman & Anderson, 1941; Freeman, 1942].

Purified antigenic complex (1·9 g.) was dispersed in 200 ml. of 0·1N acetic acid and, after 2 hr. hydrolysis at 100°, the optical rotation of the supernatant solution had become constant ($[\alpha]_D^{18°}$, +0·69° ± 0·01° (1 dm.)), indicating that hydrolysis was complete.

The following fractions were obtained:

(I) Specific polysaccharide (F0–86%), 1·326 g. = 69·8%; N, nil; ash, 4·9; R.V. 93%; $[\alpha]_D^{20°}$, +105° ± 1° (in water, $c = 1$);

(II) Conjugated protein, 0·292 g. = 15·4%; N, 7·2; ash, 5·9%; $[\alpha]_D^{20°}$, −22° ± 4° (in 0·1N NaOH, $c = 0·3$); P, 2·1%; N/P ratio, 7; a grey amorphous solid, insoluble in water, but readily soluble in dilute sodium hydroxide and reprecipitated on acidification;

(III) Lipid fraction, 0·074 g. = 3·9 %, a semi-solid, colourless fatty substance;

(IV) An ethanol residue fraction (F 86–99 %), 0·16 g. = 8·4 %; N, 2·7 %; ash, 6·4; R.V. 49 %; $[\alpha]_D^{16°}$, +15° ± 1°.

Fission of the antigenic complex, as described above, is a true hydrolytic breakdown and not merely a thermal disintegration of a colloidal complex. A specimen of antigen underwent no apparent change on heating at 100° in aqueous solution for 2 hr., and gave only 2 % of insoluble residue on centrifuging; on acidification with acetic acid and 2 hr. heating at 100° the supernatant solution broke up into its four components, as described above.

The specific polysaccharide component of the antigenic complex

The crude polysaccharide was purified by electrodialysis to remove the bulk of the ash and submitted to 2–3 fractionations from aqueous solution with ethanol.

In a typical experiment 1·83 g. of crude polysaccharide in 150 ml. water were electrodialyzed for 30 hr. in collodion membranes (Hg cathode, Pt anode, e.m.f. 100 V. d.c.), the solution concentrated *in vacuo* to about 20 ml. volume, treated with 20 mg. of norit for 30 min. at 100° and twice fractionated between the limits of 45 and 86 % ethanol concentration, giving 1·42 g. = 78 % of purified polysaccharide (F 45–86 % (2)). This gave on analysis: C, 44·6; H, 6·9; ash, 1·5 %; acetyl, nil; $[\alpha]_D^{19°}$, +103° ± 1° (in water, $c = 1$). $C_6H_{10}O_5$ requires C, 44·4; H, 6·2 %. A specimen of the electrodialyzed polysaccharide was re-electrodialyzed in 5 % solution for 70 hr., and the polysaccharide, reisolated by addition of ethanol to 86 % concentration, then gave on analysis: C, 44·5; H, 6·9; ash, 1·5 %.

It was concluded that the ash (1·5 %) was combined in the polysaccharide molecule and could not be eliminated by physical means. The pure polysaccharide precipitated with *S. typhimurium* antiserum to a dilution of 1 in 250,000. The polysaccharide gave a strongly positive Molisch's reaction but did not reduce Fehling's solution; it gave no iodine coloration. The absence of fructose residues in the molecule was demonstrated by negative Foulger's and Seliwanoff's tests. Cole's rapid furfural test, Tollens's phloroglucinol test, Bial's orcinol test and the aniline acetate test for furfural, all gave negative results, suggesting the absence of pentose residues. The polysaccharide gave a negative reaction in Tollens's naphthoresorcinol test for uronic acids: a control test on 5 mg. of glucuronic acid under the same conditions gave a strongly positive reaction. Scherer's reaction for inositol and Salkowski's [1910] modification gave negative results on 10 mg. quantities of polysaccharide; 5 mg. of inositol gave strongly positive reactions, both alone and in the presence of 10 mg. of polysaccharide.

Evidence as to the homogeneity of the polysaccharide has been obtained from: (1) attempts to separate it into fractions with different chemical properties by fractional precipitation from aqueous solution with acetone, (2) regeneration of the substance from its triacetyl derivative in an unchanged form, and (3) ultracentrifuge experiments (see Addendum).

Fractionation of polysaccharide from aqueous solution with acetone. 1·49 g. of specific polysaccharide dissolved in 40 ml. of water, giving an almost colourless, water-clear solution, free from undissolved residue, and addition of acetone to a concentration of 40 % by weight gave no opalescence, but when the acetone concentration was raised to 45 % a small amount of polysaccharide was precipitated. This was separated off, triturated with ethanol, washed twice with ether, and dried *in vacuo* over phosphorus pentoxide. Further increments of acetone were added until the material had been divided into seven fractions: F 40–45 %, F 45–50 %, F 50–55 %, F 55–60 %, F 60–65 %, F 65–75 %, and F 75–99 %. The bulk of the substance (77·6 %) was precipitated between acetone concentrations of 40 and 55 %, and only 6·2 % remained soluble in 65 % acetone. The seven fractions were compared by determination of specific optical rotation and reducing value on hydrolysis (Table 1).

Table 1. *Acetone fractionation of specific polysaccharide of* S. typhimurium

Fraction	Weight (g.)	Yield (%)	$[\alpha]_D^{20°}$ (in water, $c = 1$)	Reducing value on hydrolysis (percentage of that of glucose)
Unfractionated polysaccharide	1·4902	—	+103°	93
(1) F 40–45 %	0·1666	11·2	+101°	85
(2) F 45–50 %	0·6770	45·4	+101°	90
(3) F 50–55 %	0·3131	21·0	+101°	86
(4) F 55–60 %	0·0774	5·2	+101°	88
(5) F 60–65 %	0·0944	6·3	+105°	84
(6) F 65–75 %	0·0602	4·0	—	66
(7) F 75–99 %	0·0331	2·2	—	74

Total recovery: 95·3 % (results uncorrected for ash).

Fractions of the polysaccharide differing in solubility in acetone-water mixtures have been shown by specific rotation and reducing value determinations to be practically identical, and it may be concluded that the specific polysaccharide represents a chemical entity.

Acetylation of polysaccharide. On acetylation with acetic anhydride in pyridine solution at 70° for 1 hr., the substance (0·58 g.) yielded 65 % of theory (0·68 g.) of crude acetyl polysaccharide. The latter was purified by extraction with boiling absolute ethanol and by reprecipitation from chloroform solution with light petroleum. The two fractions precipitated by light petroleum concentrations of 63 and 85 % respectively were found to be identical on analysis, showing that the acetyl compound was homogeneous. The pure acetyl polysaccharide (triacetyl derivative) was a

colourless amorphous solid; it was insoluble, even on boiling, in absolute ethanol, methanol, ether and petroleum ether, but was readily soluble in chloroform, acetone, pyridine and acetic anhydride. It was strongly dextrorotatory, $[\alpha]_D^{18°}$, $+113° \pm 1°$ (in chloroform, $c = 1$), and gave on analysis: C, 49·9; H, 6·2; acetyl, 48·8%. $C_6H_7O_5(OC.CH_3)_3$ requires C, 50·0; H, 5·6; CH_3CO, 44·8%.

Regeneration of the polysaccharide from its acetyl derivative. The acetyl derivative was deacetylated under the conditions worked out for the deacetylation of the *Eberth. typhosa* triacetyl polysaccharide [Freeman, 1942]. 0·48 g. of the acetyl polysaccharide gave 0·228 g. (84·4% of theory) of specific polysaccharide, which gave on analysis: $[\alpha]_D^{18°}$, $+102° \pm 1°$ (in water, $c = 1$); C, 46·3; H, 6·85; acetyl, 0·9; ash, 0·7%, and precipitated to a dilution of 1 in 300,000 with *S. typhimurium* antiserum, optimum 1 in 150,000.

Acid hydrolysis of the specific polysaccharide. The optimal conditions of liberation of reducing sugars by hydrolysis of the polysaccharide were investigated as a preliminary step in the identification of the sugars present. The hydrolysis in N H_2SO_4 at 100° proceeds rapidly to about 87% (2 hr.) [Hagedorn & Jensen] and then follows a slower linear increase, reaching 93% hydrolysis after 12 hr. The unhydrolyzed polysaccharide had a reducing value, as glucose, of 1·3%. It was found that for complete hydrolysis of the polysaccharide 7 hr. heating with N H_2SO_4 at 100° was adequate.

Nature of the component reducing sugars

The specific polysaccharide has been shown to consist predominantly of anhydrohexose units; ketose, pentose and uronic acid residues were absent. *d*-Galactose has been shown to be present in the antigenic complex from which the polysaccharide was derived [Freeman *et al.* 1940].

The hydrolysate from 0·971 g. of specific polysaccharide, freed from H_2SO_4 and concentrated to 25 ml., gave $\alpha_D^{19°}$, $+1·12°$ (1 dm.); total apparent glucose = 0·8115 g. (83·6% of the polysaccharide). If the reducing constituents had a reducing value equal to that of glucose, the 'apparent specific rotation' of the mixture was $[\alpha]_D^{19°}$, $+34·5°$.

d-*Mannose-phenylhydrazone.* One-half of the total neutral hydrolysate was treated with 0·2 g. phenylhydrazine and 0·4 g. 25% acetic acid [van der Haar, 1920; Freeman, 1942]; mannose phenylhydrazone crystallized out on standing at 0°, yield 0·1486 g., corresponding to a mannose content of 20·4% of the specific polysaccharide. The crude product was recrystallized from boiling aqueous ethanol (60% w/w) and gave *d*-mannose phenylhydrazone, colourless microcrystalline plates, m.p. 195° (186° on slow heating), unchanged on admixture with an authentic specimen. Found: C, 53·2; H, 6·7; N, 10·3%. $C_{12}H_{18}N_2O_5$ requires C, 53·3; H, 6·7; N, 10·4%.

d-*Galactose.* The excess phenylhydrazine was removed as benzaldehyde phenylhydrazone [Morgan, 1938], the filtrate concentrated *in vacuo* to dryness and the residue of sugars taken up in 2 ml. 90% acetic acid and the solution allowed to crystallize at 0°. 0·053 g. of crystalline *d*-galactose (10·8% of the polysaccharide) separated out. The product reduced Fehling's solution (m.p. 161°, mixed m.p. with authentic *d*-galactose (m.p. 164°) was 161°, $[\alpha]_D^{19°}$, $+80° \pm 2°$ (in water, $c = 1$)). A specimen (0·038 g.) yielded 90% (0·054 g.) of *d*-galactose methylphenylhydrazone, m.p. 190°, unchanged on admixture with an authentic specimen.

A small further yield of *d*-galactose (2·0% of the polysaccharide) as the methylphenylhydrazone was obtained from the filtrate after removal of the crystalline *d*-galactose by treatment with methylphenylhydrazine (asym.) [Neuberg, 1907; Lüdtke, 1929; Freeman, 1942]. Found: C, 55·0; H, 7·1; N, 10·0%. $C_{13}H_{20}N_2O_5$ requires C, 54·9; H, 7·1; N, 9·9%.

d-*Glucose*-p-*nitrophenylhydrazone.* Excess methylphenylhydrazine was eliminated as described above, the sugar-containing filtrate evaporated to dryness *in vacuo* and the residue taken up in 2 ml. absolute methanol and treated with *p*-nitrophenylhydrazine (0·1 g.) [van Ekenstein & Blanksma, 1903; Reclaire, 1908; Freeman, 1942]. Yellow crystalline *d*-glucose-*p*-nitrophenylhydrazone was obtained (0·044 g., 5·2% of glucose), m.p. 184°, unchanged on admixture with authentic hydrazone. Found: C, 45·7; H, 5·6; N, 13·3%. $C_{12}H_{17}O_7N_3$ requires C, 45·7; H, 5·4; N, 13·3%.

The isolations of sugars, as substituted phenylhydrazones, under conditions in which the derivative of a single hexose is obtained at each stage, provide conclusive proof of the co-existence of *d*-glucose, *d*-mannose and *d*-galactose as constituents of the specific polysaccharide. The losses of sugars in the intermediate removal of excess of substituted hydrazines with benzaldehyde are, however, sufficiently great to lower significantly the yields of hydrazones.

In a further series of experiments the neutralized hydrolysate from 1·45 g. of polysaccharide gave 0·274 g. (18·9%) of crystalline *d*-galactose. After recrystallization from 90% acetic acid, the m.p. was 165°, unchanged on admixture with authentic *d*-galactose; the initial and equilibrium rotations (in water, $c = 1·7$) were: $[\alpha]_D^{18°}$, $+143° \pm 1°$ (after 3 min.); $+80·5° \pm 1°$ (after 24 hr.) (these agree closely with the recorded values for α-*d*-galactose, $+144·0° \rightarrow +80·5°$ [Hudson & Yanovsky, 1917]) and $+80·4°$ (at equilibrium) [Parcus & Tollens, 1890].

The residual sugars in solution were heated under reflux with *p*-nitrophenylhydrazine (1 g.) in methanol. *d*-Mannose-*p*-nitrophenylhydrazone (0·473 g., 18·6% mannose in terms of the polysaccharide) crystallized out as pale yellowish brown crystals from the boiling solution, m.p. 190°, unchanged on admixture with authentic *d*-mannose-*p*-nitrophenylhydrazone. On admixture with *d*-glucose-*p*-nitrophenylhydrazone (m.p. 184°) the mixed m.p. was 175°. $[\alpha]_D^{14°}$, $+86° \pm 1°$ (in pyridine, $c = 1$), which agreed with that of the authentic hydrazone ($[\alpha]_D^{15°}$, $+87° \pm 1°$).

0·6234 g. (24·6% as *d*-glucose) of *d*-glucose-*p*-nitrophenylhydrazone separated out from the filtrate at 0° after removal of *d*-mannose-*p*-nitrophenylhydrazone.

Thus 18·9% of *d*-galactose has been isolated from the specific polysaccharide as the free sugar, in addition to yields of substituted hydrazones equivalent to 24·6% *d*-glucose, and to 20·4% *d*-mannose (20·4% as the phenylhydrazone, and in another experiment 18·6% as the *p*-nitrophenylhydrazone). Control experiments under the same conditions with similar con entrations of single pure hexoses have

shown that pure *d*-glucose gives 79·6% of the theoretical yield of the *p*-nitrophenylhydrazone and pure mannose 94·9% of the theoretical yield of *d*-mannose phenylhydrazone [Freeman, 1942]. On this basis the actual hexose contents of the specific polysaccharide are estimated to be: *d*-glucose, 31; *d*-mannose, 21·5; *d*-galactose, 18·9; total hexose, 71·4%. The latter approaches reasonably close to the reducing value of 90% (as apparent glucose) on hydrolysis of the polysaccharide.

A useful confirmation of the relative proportions of the three hexoses present in the polysaccharide hydrolysates is afforded by a comparison of the observed optical rotation of a neutralized hydrolysate with that calculated from the reducing value of the solution (Hagedorn & Jensen) in terms of apparent glucose. The hydrolysate described above had $\alpha_D^{18.5°}$, +1·12° (1 dm.), total volume 25 ml. and total apparent glucose, 0·8115 g. In such a solution, containing 31% glucose, 21·5% mannose and 18·9% galactose, the rotation of the *d*-glucose component is +0·53°, that of the mannose component +0·10° and that of the galactose +0·50°, giving a total calculated rotation (1 dm.) of +1·13°, which agrees closely with the observed value.

The conjugated protein component of the antigenic complex

The conjugated protein fraction gave positive biuret, Millon's and Sakaguchi's reactions, showing the presence of tyrosine and arginine residues. Ehrlich's diazo reaction for histidine was also positive.

The crude substance (0·63 g.) was purified by three precipitations from 0·1N NaOH with 4N HCl [Freeman & Anderson, 1941] and gave 76% (0·48 g.) of purified conjugated protein, N, 6·2; ash, 5·1%.

Tryptic digestion of the conjugated protein. 0·0725 g. of the conjugated protein was freshly precipitated by acidifying an alkaline solution of the substance, and suspended in 15 ml. of borate buffer, pH 8·4; trypsin (0·003 g.) was added and the digest incubated at 37°. After 5 days' digestion only 5·1% of the total N had been liberated as amino-N, and 17·7% after 14 days (Van Slyke). The substance was, therefore, only slowly and very incompletely attacked by trypsin.

Acid hydrolysis of the conjugated protein. 0·01 g. quantities of the conjugated protein were hydrolyzed with 1 ml. 10N formic acid containing 3·6% HCl in sealed tubes at 100° for various periods of time and the amino-N liberated determined by the Van Slyke method [Morgan & Partridge, 1940*a*; Freeman & Anderson, 1941]. After 2–4 hr. hydrolysis the bulk of the conjugated protein had dissolved, giving a pale brown solution, although traces of insoluble residue were still present after 8 hr. heating. The amino-N, liberated from the conjugated protein, progressively increased to 50·5% of the total N after 4–8 hr. hydrolysis and at this stage liberation of amino-acids had reached a maximum.

The 86% ethanol-soluble fraction of the antigenic complex. In its crude state this fraction was a colourless, very hygroscopic solid, which dissolved readily in water but was insoluble in chloroform and acetone; it gave a strongly positive Molisch's reaction, showing the presence of carbohydrate residues, but negative ninhydrin and biuret reactions, from which it was concluded that the nitrogen was not present as amino-acids or peptides. The fraction reduced Fehling's solution on being boiled with it for about 20 sec. It gave an immediate precipitate with 2% phosphotungstic acid in 5% H_2SO_4. On hydrolysis the substance yielded 21·5% of volatile fatty acid (calculated as acetyl) and a trace of glucosamine (1%). The nature of this fraction and its role in the antigenic complex are not yet understood. The compound may be a partially acetylated polysaccharide. The bulk of the volatile fatty acid component (acetyl) of the antigenic complex is present in the 86% ethanol-soluble fraction. The substance has no power of specific precipitation with *S. typhimurium* antiserum.

The lipid components of the antigenic complex

The lipid fraction liberated on acetic acid hydrolysis constitutes only 3–4% of the antigen. By dissociation of the antigenic complex with formamide a part of this lipid fraction (0·9% of the antigen) is liberated. Although other workers have isolated much larger amounts of lipid components from bacterial antigens [Morgan & Partridge, 1940*a*; Soru & Combiesco, 1940; Boivin & Mesrobeanu, 1938], our *S. typhimurium* antigen does not appear to contain more than about 5% of this component.

0·145 g. of the lipid fraction was separated by the usual methods, into a mixed fatty acid fraction (colourless solid, 0·063 g., 43%), a fraction insoluble in cold acetone, which was probably a phospholipin (0·09 g. = 6·4%), and 0·04 g. (28%) of neutral fatty substance. Insufficient of the lipid component was available for further investigation.

Preparation of the specific polysaccharide directly from the dried bacterial bodies

As in the case of *Eberth. typhosa* Ty 2, the specific polysaccharide of *S. typhimurium* may be extracted directly from the dried bacterial cells by means of dilute acetic acid at 100° as described by Freeman [1942] [cf. White, 1929, and Morgan, 1936].

28 g. of the dried cells were extracted three times for 1½ hr. at 100° with 400 ml. lots of 0·1N acetic acid. The successive extracts had optical rotations of +0·57°, +0·12° and −0·06° (1 dm., 18°), and yielded 4·3 g. (15·4%) of colourless crude polysaccharide (N, 5·9; ash, 25·9; R.V. 84%; $[\alpha]_D^{16°}$, +67°±4° (in water, *c* = 1)). The substance precipitated with *S. typhimurium* antiserum to a dilution of 1 in 300,000.

The polysaccharide was purified as described by Freeman [1942]; 1·88 g. of the crude material gave 0·564 g. (30%) of pure polysaccharide, which gave on analysis: C, 45·4; H, 6·8; inorganic P, 0·1%; organic P, 0·3%; N, nil. The yield of pure polysaccharide was 4·6% of the dried bacterial bodies, as compared with a yield of about 4·8% in terms of the dried bacterial bodies, by hydrolysis of the isolated antigenic complex.

A rough strain of *S. typhimurium* (100 g. dried bodies) was twice extracted with 1000 ml. lots of 0·1 N acetic acid for 1½ hr. at 100°. The extracts, which had optical rotations of +0·15° and +0·06° (1 dm., 17°), were found to contain no significant amount of the specific polysaccharide. A fraction F 50–86% (1) was obtained (4·2%), which gave only a trace of precipitate with *S. typhimurium* antiserum at a dilution of 1 in 15,000. The specific polysaccharide tested at the same time precipitated to a dilution of 1 in 300,000.

Preparation of an undegraded specific polysaccharide

Morgan & Partridge [1941] have shown that by dissociation of the antigenic complex of *Bact. dysenteriae* (Shiga) with dilute NaOH, and by means of extraction with concentrated aqueous solutions of phenol, a specific non-antigenic polysaccharide was obtained, which differed markedly, in chemical and physical properties, from the ('degraded') polysaccharide obtained by means of hydrolysis with hot dilute acetic acid, and possessed the power, not shared by the 'degraded' polysaccharide, of recombining with the conjugated protein component of the antigen to produce antigenically active complexes. The antigenic complex of *S. typhimurium* has been submitted to fractional degradation by methods analogous to those described by Morgan & Partridge.

(1) *By sodium hydroxide dissociation.* The *S. typhimurium* antigen (1·34 g.), in 100 ml. of water, was treated with NaOH to a concentration of 0·05 N at 0°, as described by Morgan & Partridge; 1·13 g. (85%) of 'recovered antigen' was obtained, and this did not differ significantly from the untreated antigen in its solubility, appearance in solution and qualitative reactions. Only 0·007 g. (0·5%) of material was recovered from the alkaline alcoholic supernatant solution. This gave a negative biuret reaction. It was clear that 0·05 N sodium hydroxide had no significant dissociating effect on the antigenic complex.

'Recovered antigen' from the previous experiment was treated with 0·25 N NaOH. An ethanol precipitated fraction (F 68% (2), 1·01 g. = 94%; N, 1·2; ash, 2·2%; $[\alpha]_D^{18°}$, +104° ±1° (in formamide, $c = 1$)) and a protein-like fraction soluble in alkaline ethanol, were obtained. The latter (0·041 g. = 3·9%; N, 9·0%; ash, nil) was a colourless, amorphous solid, which was insoluble in water but dissolved freely in 0·1 N NaOH and was slowly precipitated from alkaline solution on acidification with 2 N HCl. The substance gave positive biuret, Sakaguchi's and Ehrlich's diazo reactions, indicating the presence of peptides and arginine and histidine residues. The compound was amphoteric; it dissolved slowly in N acetic acid at 40° as well as in dilute alkali. After reprecipitation from alkaline solution and from solution in N acetic acid, the substance remained virtually unchanged, N = 9·3%.

The recovered 'polysaccharide' (F 68% (2), 0·91 g.) was submitted to two further dissociations with 0·25 N alkali and gave 0·68 g. (75%) of 'undegraded polysaccharide' (F 68% (4), N, 0·5%), equivalent to 60% of the undissociated antigen. The undegraded polysaccharide gave aqueous solutions very much less opalescent than those of the original antigenic complex. 0·021 g. (2·3%) of amphoteric protein fraction was also obtained from the third and fourth alkali treatments of the antigen.

In order to establish the homogeneity of the undegraded polysaccharide, F 68% (4) (0·62 g.) was fractionally precipitated from aqueous solution with ethanol. Three fractions were isolated:

(*a*) F 55–60% (0·56 g. = 90·0%; C, 47·1; H, 7·3; acetyl 3·5; ash, 6·3; R.V. 73%; $[\alpha]_D^{16°}$, +112° ±1°; limit of precipitation with antiserum, 1 in 300,000, optimum 150,000),

(*b*) F 60–70% (0·004 g. = 0·7%), and

(*c*) F 70–99% (0·048 g. = 7·8%; R.V. 75%; limit of precipitation with antiserum 1 in 150,000; optimum 1500), and it was concluded from the results that the alkali-dissociated polysaccharide, F 68% (4), was chemically homogeneous. Further evidence on the homogeneity of this substance was provided by ultracentrifugal sedimentation (see Addendum).

(2) *By extraction of the antigenic complex with concentrated aqueous phenol solutions. Salmonella typhimurium* antigenic complex (0·984 g.) was repeatedly extracted with 80% aqueous phenol and 95% aqueous phenol; the former yielded 0·027 g. (2·7%) of amphoteric protein and the latter none. The 'recovered polysaccharide' (1) (0·75 g. = 76·5%; $[\alpha]_D^{18°}$, +81° ±3° (in formamide, $c = 1$)) was an amorphous substance which gave very opalescent aqueous solutions. Recovered polysaccharide (1) (0·722 g.) was three times re-extracted with 80% phenol and gave 0·621 g. (86·0%) of recovered polysaccharide (2) (N, 0·5%). The latter gave very opalescent aqueous solutions similar to those of the original antigen, and was precipitated from solution by 2% phosphotungstic acid in 5% H_2SO_4.

In order to investigate the homogeneity of recovered polysaccharide (2), the material (0·559 g.) was fractionally precipitated from aqueous solution (50 ml.) with ethanol. The properties of the fractions isolated are summarized in Table 2. Fraction F 50–60% gave on analysis, C, 49·1; H, 7·9; ash, 5·5%.

Table 2. *Analytical data for fractions of* S. typhimurium *'undegraded polysaccharide' prepared by phenolic extraction*

	Insoluble residue	Ethanol fractions of 'polysaccharide'		
		F 50–60%	F 60–65%	F 65–99%
Weight (g.)	0·069	0·298	0·112	0·019
Yield (%)	12·3	53·4	20·1	3·5
N (%)	—	0·9	—	—
Reducing value on hydrolysis (percentage of that of glucose)	—	66	72	—
Dilution limit of precipitation with *S. typhimurium* antiserum (1 part dissolved in:)	—	1,000,000	1,000,000	—
Dilution giving optimal precipitation (1 part dissolved in:)	—	300,000	300,000	—

Total recovery: 89·3%.

Recombination of Salmonella typhimurium *'undegraded polysaccharides' with proteins*

Antigenically active artificial complexes of the specific polysaccharide and conjugated protein components of *Bact. dysenteriae* (Shiga) have been obtained by Morgan & Partridge [1940 *a*, *b*] by dissolving the isolated components in formamide and precipitating the complex with ethanol, and later, using an undegraded specific polysaccharide, simply by mixing the components of the complex in alkaline solution [Morgan & Partridge, 1941]. The observation that bacterial polysaccharides possess the power of forming acid-soluble complexes with proteins, which are normally insoluble in aqueous solution at acid reaction, has been confirmed and extended to the undegraded polysaccharide of *S. typhimurium*. If a solution of the undegraded polysaccharide and an alkaline solution of the protein are mixed and acidified with acetic acid, a soluble, stable complex is formed, and the protein is no longer precipitated by the addition of trichloroacetic acid or other protein precipitants, provided that an excess of the protein above that equivalent to the combining power of the undegraded polysaccharide has not been used.

By this means the 'undegraded polysaccharides' prepared by alkaline dissociation and by phenolic extraction of the *S. typhimurium* antigenic complex were found to form acid-soluble complexes with the conjugated proteins of *S. typhimurium* and *Eberth. typhosa* Ty 2. When the specific polysaccharide (acetic acid hydrolytic product) replaced the undegraded polysaccharide, the admixed proteins were immediately precipitated by acid. The alkali-dissociated undegraded polysaccharide had the power of rendering soluble at least an equal weight of conjugated protein (*S. typhimurium*), but the phenol-extracted 'polysaccharide' was saturated by more than 40% of protein; this shows that the two 'undegraded polysaccharides' are clearly different.

The alkali-dissociated undegraded polysaccharide formed trichloroacetic acid-soluble complexes on admixture in alkaline solution with edestin, human serum pseudoglobulin, casein, gliadin and rabbit myosin. When the polysaccharide was mixed with rabbit myosin in neutral salt solution, no acid-soluble complex was formed. Control experiments with *S. typhimurium* conjugated protein and with mixtures of the above proteins with degraded polysaccharide, gum arabic or soluble starch, all gave negative results.

The undegraded polysaccharide lost its power of combining with the conjugated protein after 1 hr. heating in 0·1N acetic acid solution at 100°, but there was no appreciable loss of this activity after 30 min. at 50°, or 5 min. at 100°, in acetic acid. After 1 hr. hydrolysis at 100° with 0·1N acetic acid the undegraded polysaccharide solution had lost its opalescence and had deposited a trace of precipitate (2·1%). At this stage the hydrolysate contained two polysaccharides, which were separated by fractional precipitation with ethanol: (*a*) (F 0-86%), 80·2%, $[\alpha]_D^{19°}$, $+100° \pm 1°$; this substance was identical with the degraded polysaccharide prepared by acetic acid hydrolysis of the antigenic complex; and (*b*) (F 86–99%), 0·032 g. = 10·6%; acetyl, 13·2%; insufficient of this component was obtained for adequate purification, but the evidence available strongly suggests that the substance, which was a very hygroscopic acetyl polysaccharide, consisted predominantly of the acetyl polysaccharide previously isolated directly from the antigenic complex.

An artificial complex of the undegraded polysaccharide and the conjugated protein behaved, on heating at 100° in 0·1N acetic acid solution, in a manner identical with that of the native antigenic complex. An artificial complex of undegraded polysaccharide and horse serum albumin was stable in neutral solution at 100° for 2 hr., giving no precipitation of the albumin.

Combination of undegraded polysaccharide and horse serum albumin. Determination of saturation value. The undegraded polysaccharide was found to form a trichloroacetic acid-soluble complex with horse serum albumin when the two components were mixed either in neutral or in alkaline solution at 30°. (The horse serum albumin was a specimen of lyophil-dried protein kindly supplied by Dr A. G. Ogston; see Addendum.) When the ratio protein/polysaccharide did not exceed 3·5, trichloroacetic acid-soluble complexes were formed, but increase of protein above this proportion resulted in heavy precipitates on addition of trichloroacetic acid. In the neighbourhood of the equivalence zone addition of the precipitant produced very opalescent solutions, from which a small amount of protein separated out on standing.

The nitrogen contents of the precipitates and supernatant solutions obtained from several undegraded polysaccharide-albumin mixtures, by treatment with 20% trichloroacetic acid, were determined after drying by the Dumas method. It was found that when the ratio protein/polysaccharide did not exceed 3·5, practically the whole of the protein remained in the supernatant solution, but that when this figure was exceeded the whole of the protein was precipitated, practically none remaining in the supernatant solution.

Titration curves of artificial polysaccharide-protein complexes

In order to investigate the changes which take place in the solubility and buffering power of proteins in the presence of *S. typhimurium* undegraded polysaccharide, (*a*) horse serum albumin and

(*b*) *S. typhimurium* conjugated protein, alone and in the presence of the undegraded polysaccharide, were titrated at 19° with 0·01N HCl and NaOH, and the pH determined at intervals with a glass electrode and valve electrometer pH meter.

The following solutions were titrated:

(i) 5 ml. 1% aqueous albumin + 4 ml. water.
(ii) 5 ml. 1% aqueous albumin + 4 ml. 0·5% polysaccharide.
(iii) 5 ml. 1% conjugated protein + 4 ml. water.
(iv) 5 ml. 1% conjugated protein + 4 ml. 0·5% polysaccharide.
(v) 5 ml. water + 4 ml. 0·5% polysaccharide.
(vi) 9 ml. distilled water.

The conjugated protein solution was prepared by dissolving the substance (0·14 g.) in 2·5 ml. 0·1N NaOH, adding 1 ml. 0·1N HCl to the solution and diluting to 14 ml. with water.

Between pH 5·8 and 4·5 in titration (iii) each successive addition of acid produced a slight precipitate, which rapidly redissolved on stirring; at pH 4·47 a permanent precipitate of conjugated protein appeared which separated out on standing, leaving a clear supernatant solution. In the presence of the undegraded polysaccharide (titration (iv)) there was no change in the appearance of the solution and no precipitation of protein.

The undegraded polysaccharide had no appreciable buffering power; titrations (v) and (vi) gave practically identical results. The presence of the undegraded polysaccharide brought about no significant change in the buffering power of serum albumin (Fig. 1) on the acid side of pH 6, but in the range of pH 6–10 there was an appreciable increase of buffering power. The displacement of the serum albumin curve in the presence of the undegraded polysaccharide occurred in the same pH range and in the same sense as that produced by formaldehyde [Prideaux & Woods, 1933], but was not nearly as great. In the experiments of Prideaux & Woods, 5–6 ml. more 0·1N alkali were required to bring the albumin to pH 9 in the presence of formaldehyde, whereas only 12 ml. more 0·01N alkali would be required to bring a corresponding quantity of albumin to pH 9 in the presence of undegraded polysaccharide. In the titration curves of *S. typhimurium* conjugated protein (Fig. 2) there was a marked decrease of buffering power, in the presence of the polysaccharide, in the pH range 4–7, which coincided with the range of solubility of the conjugated protein on the acid side of neutrality.

Katchalsky [1941] observed depression of the pH when glucose was added to solutions of various amino-acids and peptides, from which he has determined the equilibrium constants of the interaction of the two components.

It is clear that interaction or combination takes place between the protein and undegraded polysaccharide molecules, with the production of complexes which behave towards acids and alkalis differently from the untreated protein. Nothing is known of the nature of the protein-polysaccharide bond, but this may be salt-like, or possibly of the Schiff's base type through a polysaccharide alde-

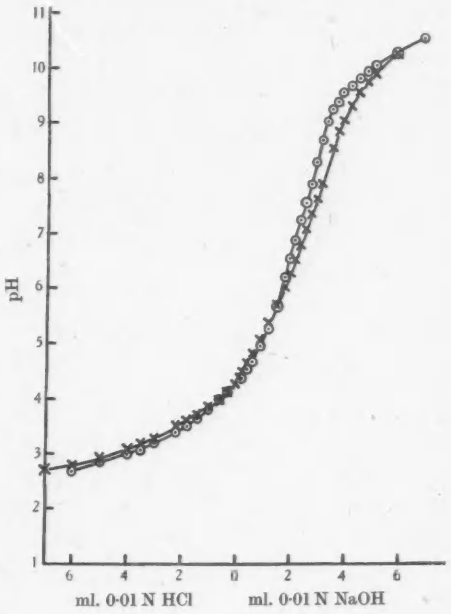

Fig. 1. Titration curve of serum albumin in presence and absence of *Salmonella typhimurium* undegraded polysaccharide. ⊙—⊙ Serum albumin, ×—× serum albumin + polysaccharide.

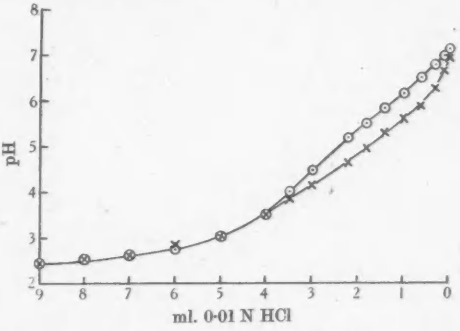

Fig. 2. Titration curve of *Salmonella typhimurium* conjugated protein in presence and absence of undegraded polysaccharide. ⊙—⊙ Conjugated protein, ×—× conjugated protein + polysaccharide.

hydic group and an amino-group in the protein. The evidence available suggests that the combination is not analogous to the interaction of formaldehyde with amino-groups, which involves a considerable pH shift and alteration of the pK's of the amino-groups, whereas in the formation of the undegraded polysaccharide-protein complex there is a very small pH shift, and the pK's of the amino-groups are not seriously changed despite the formation of an apparently stable complex, in which the polysaccharide renders the protein more soluble in the presence of acid precipitants. These conclusions regarding the existence of an artificial protein-polysaccharide complex are confirmed by ultracentrifugal determination of sedimentation constants (see Addendum).

Antigenicity of undegraded polysaccharides and an artificial polysaccharide-conjugated protein complex

The following fractions were tested for antigenicity in rabbits:

(i) Undegraded polysaccharide (phenol-dissociated product).

(ii) Undegraded polysaccharide (alkali-dissociated product).

(iii) 'Degraded' specific polysaccharide.

(iv) *Salmonella typhimurium* conjugated protein (dissolved in phosphate buffer (pH 8·4) plus saline).

(v) Artificial polysaccharide-conjugated protein complex (70 mg. undegraded polysaccharide, alkali dissociated, +20 mg. conjugated protein).

(vi) *Salmonella typhimurium* antigenic complex. (The author is indebted to Dr A. R. Martin for the rabbit immunizations.) The rabbit antisera were tested for the presence of specific precipitins against solutions of the homologous antigens, and for their power of agglutinating killed O-suspensions of *Salmonella typhimurium* (Table 3).

The phenolic-dissociation product and the artificial polysaccharide-conjugated protein complex were powerfully antigenic, and gave rise to antisera which precipitated to high dilutions with the homologous antigens, and agglutinated *S. typhimurium* suspensions to about the same dilution as an antiserum prepared against the purified antigenic complex. The alkali-dissociation product (undegraded polysaccharide) and the degraded specific polysaccharide, on the other hand, had no detectable antigenic activity. The phenolic- and alkali-dissociated products, and the degraded polysaccharide, precipitated with an antiserum prepared against the original antigenic complex to dilutions of 1 in 1,000,000 (optimum 1 in 150,000), 1 in 300,000 (optimum 1 in 15,000) and 1 in 300,000 respectively. *S. typhimurium* conjugated protein was feebly antigenic, giving rise to homologous precipitins, but sera 296 A and B were free from *S. typhimurium* agglutinins and gave no precipitates with the original antigenic complex. By combination of the inactive undegraded polysaccharide and the feebly antigenic conjugated protein, which had no *S. typhimurium* specificity, a powerfully antigenic artificial complex was obtained, which gave rise to antisera which precipitated the homologous antigen and the original antigenic complex (to dilutions of 1 in 600,000, optimum 1 in 15,000) and agglutinated *S. typhimurium* suspensions.

The phenolic-dissociated undegraded polysaccharide precipitated with antisera prepared against the parent antigenic complex in a manner almost identical with that of the alkali-dissociated undegraded polysaccharide, had similar chemical and physical properties and also possessed the power of recombining with the conjugated protein com-

Table 3. *Antigenicity of fractions from* S. typhimurium

Material used as antigen	Rabbit no.	Precipitation test. Dilution of homologous antigen 1 in 1500	15,000	150,000	300,000	Agglutination test. Dilution of immune serum 1 in 40	80	160	320	640	1280
(i) Undegraded polysaccharide (phenol-dissociated product)	263A	+ +	+ + + + (optimum)	+ + +	+ + +	4	4	4	3	1	0
	263B	+ +	+ + + (optimum)	+ + +	+	4	4	4	3	1	0
(ii) Undegraded polysaccharide (alkali-dissociated product)	266A	—	—	—	—	0	0	0	0	0	0
	266B	—	—	—	—	0	0	0	0	0	0
(iii) 'Degraded' specific polysaccharide	265A	—	—	—	—	0	0	0	0	0	0
	265B	—	—	—	—	0	0	0	0	0	0
(iv) *S. typhimurium* conjugated protein	296A	+ +	+ +	+ +	—	0	0	0	0	0	0
	296B	+ + (optimum)	+ +	+ +	—	0	0	0	0	0	0
(v) Artificial polysaccharide-conjugated protein complex	298A	+ +	+ + +	+	—	4	4	4	3	1	0
	298B	+ + +	+ + + + (optimum)	+ +	+	4	4	4	2	1	0
(vi) *S. typhimurium* antigenic complex	230	+	+ + + (optimum)	+ + +	+ +	4	4	4	4	2	0

4, complete agglutination; 3, almost complete agglutination; 2, incomplete agglutination; 1, trace of agglutination; 0, no agglutination.

ponent, though not so powerfully as the alkali dissociation product. It was clearly different from the latter, as shown by its antigenic properties in rabbits, though this property may be traceable to residual small amounts of the antigenic complex still undecomposed by repeated phenolic extractions.

DISCUSSION

In its nature and properties the antigen of *S. typhimurium* shows a close similarity to the corresponding complex from *Eberth. typhosa* Ty 2. Both antigens exist as polysaccharide-conjugated protein-lipid complexes, which are readily broken down into their components by hydrolysis. There is also a fourth component which has not yet been isolated in a pure condition, but is described provisionally as a partially acetylated polysaccharide. When the antigenic complex is degraded, its antigenic properties are lost but the polysaccharide component retains the specific properties of the complex. The latter undergoes partial degradation, with the elimination of one or more of its components, on treatment with formamide, dilute alkali and concentrated aqueous phenol. Dilute alkali and phenol liberate an amphoteric protein, which differs from the conjugated protein in being soluble at acid reactions, and an 'undegraded polysaccharide', intermediate in properties between the antigenic complex and the specific polysaccharide. The undegraded polysaccharide differs very significantly from the specific polysaccharide, in possessing the power of combining with proteins to form stable complexes, and there is evidence that it consists of a complex of the specific polysaccharide and the acetyl polysaccharide.

Previous workers have isolated antigenic complexes from *S. typhimurium* but some of the earlier preparations were undoubtedly contaminated with materials derived from the culture media and evidence of their chemical homogeneity is not always available. Raistrick & Topley [1934] prepared antigenic fractions which contained 4–6·5% nitrogen and gave 23·1–37·8% reducing sugars on hydrolysis. Topley *et al.* [1937] describe an antigenic fraction which contained 4·2–4·8% N. Boivin's antigen from the same organism contained 1·83–2·20% N; R.V. 40% on hydrolysis [Boivin & Mesrobeanu, 1934]. At an earlier stage of this investigation, Freeman *et al.* [1940] obtained the antigen for the first time from organisms grown on a synthetic medium; it contained N, 4·2%, and gave 50% of reducing sugars on hydrolysis, $[\alpha]_{5461}$, +76°. These data relate to the crude fraction which was the starting material in the ethanol and ammonium sulphate fractionations in the present work. These figures illustrate the difficulties of obtaining pure antigenic fractions from the bacterial cell, and emphasize the need for careful fractionation of the primary extraction products.

In their behaviour on hydrolysis, and in the properties of their conjugated protein and specific polysaccharide components, the antigenic complexes of *S. typhimurium* and *Eberth. typhosa* display a marked similarity, but there are considerable quantitative differences of nitrogen content, reducing value on hydrolysis and specific optical rotation. The conjugated proteins of the two antigenic complexes are markedly similar in their physical properties, N contents, constituent amino-acids and liberation of amino-nitrogen on tryptic digestion and acidic hydrolysis.

As was to be expected from the immunological data for the two organisms, cross precipitation reactions between the specific polysaccharides of *S. typhimurium* and *Eberth. typhosa* Ty 2 were negative; the chemical data confirm that the two substances are different. Both polysaccharides, however, consist entirely of the same hexose units (*d*-glucose, *d*-mannose and *d*-galactose), which are present in approximately the same relative amounts. Differences in the structural arrangement of the constituent sugars, which may account for the serological dissimilarity of the two specific polysaccharides, provide an interesting subject for investigation when more of the specific substances become available.

The work of Morgan & Partridge [1941] revealed new methods of degradation of the parent antigenic complex, which do not involve high temperatures or extremes of acidity. These have been used successfully to prepare undegraded polysaccharides from *S. typhimurium*, which were intermediate in properties and composition between the parent antigenic complex and the degraded polysaccharide. The undegraded polysaccharides possess the remarkable property of forming stable complexes with proteins, from which the latter are not precipitable by acid protein precipitants. The alkali- and phenol-dissociation products (undegraded polysaccharides) display an important difference in that the former is non-antigenic, whilst the latter still stimulates formation of precipitins in rabbits. This antigenicity is thought to be due to residual traces of undecomposed antigenic complex.

Morgan & Partridge also showed how the polysaccharide and conjugated protein components of *Bact. dysenteriae* (Shiga), lacking specific antigenicity, could be recombined to form complexes which gave rise to powerful specific antibodies on injection into animals. This has been confirmed and extended to *S. typhimurium*; mixture of the non-antigenic alkali-dissociated polysaccharide and the conjugated protein (feebly antigenic but with no *S. typhimurium* specificity) in alkaline solution, followed by acidification, produced a powerfully antigenic

complex which gave rise to *S. typhimurium* precipitins and agglutinins on injection into rabbits. Ultracentrifugal studies of mixtures of the undegraded polysaccharide with albumin also clearly showed the presence of a new complex.

SUMMARY

1. By means of ethanol and ammonium sulphate fractionation from aqueous solution the antigenic complex of *Salmonella typhimurium* has been obtained in a form which is believed to represent a chemical entity.

2. On acetic acid hydrolysis the antigenic complex is broken down into four components: (*a*) a specific polysaccharide (69 %), (*b*) an insoluble conjugated protein (16 %), (*c*) a mixed lipid fraction (3–4 %) and (*d*) an alcohol-soluble acetyl-polysaccharide (about 8 %).

3. The specific polysaccharide is non-antigenic. It is dextrorotatory, $[\alpha]_D^{19°}$, $+103° \pm 1°$; it is free from nitrogen and yields 93 % of reducing sugars on acidic hydrolysis. The polysaccharide is free from ketose, pentose and uronic acid residues, and yields 31 % *d*-glucose, 21·5 % *d*-mannose and 19 % *d*-galactose on hydrolysis.

4. The antigenic complex has also been dissociated by means of precipitation from weakly alkaline solution, yielding a small amount of an amphoteric protein, and a non-antigenic 'undegraded polysaccharide' which reacts specifically with *Salmonella typhimurium* antiserum to high dilutions. This polysaccharide has the power of forming complexes with proteins such that the protein becomes no longer precipitable by acidic protein precipitants.

The author wishes to thank Prof. Sir Robert Robinson for his interest in this work and Imperial Chemical (Pharmaceuticals) Limited for permission to publish this account of it.

ADDENDUM

Examination in the Ultracentrifuge

By A. G. OGSTON (with technical assistance by Mrs P. M. Hodson),
Department of Biochemistry, Oxford

Various samples derived from the antigen of *Salmonella typhimurium* were examined in a Svedberg oil-turbine ultracentrifuge, by the 'diagonal schlieren' method of Philpot [1938]; where it was possible, the total refractive increments of the dissolved substances were measured, and the refractive increments appearing in different boundaries determined by integration of areas in the diagrams [Philpot, 1939].

(1) '*Degraded*' *polysaccharide:* 1 % solution unbuffered, in 0·1M NaCl; $S^w_{20} = 1·25 \times 10^{-13}$ is close to the value given by Philpot [1942] for the polysaccharide derived from *Eberth. typhosa* Ty 2. The form of the sedimentation diagram (Fig. 3) indicates fair homogeneity and probably a not very asymmetric shape of particle (less so than that of *Eberth. typhosa* polysaccharide): if we assume a partial specific volume of 0·62, this gives a minimum molecular weight of 3600. Only 80 % of the total refractive increment appears in the boundary, which shows that there is some non-sedimenting or highly heterogeneous material.

(2) *Undegraded polysaccharide:* 0·5 % in 0·01M phosphate buffer, pH 6·8 and 0·2M NaCl: this presents a very different picture. There are at least three molecular species present of $S^w_{20} = 13·1$, 7·6 and $1·72 \times 10^{-13}$ respectively: the diagram (Fig. 3) indicates also some rather heterogeneous material of sedimentation constant greater than 13×10^{-13}, and possibly a small component of sedimentation constant less than $1·72 \times 10^{-13}$. The form of boundary shown by the component of $S^w_{20} = 7·6$ suggests that its particles are asymmetric. The component of $S^w_{20} = 1·72$ may be identical with the 'degraded' polysaccharide.

(3) In view of the remarkable power of the undegraded polysaccharide to prevent the precipitation of proteins by trichloroacetic acid, a solution of it with crystalline horse serum albumin was compared with solutions of the polysaccharide and of albumin separately. The solution was 0·5 % with respect to polysaccharide, 1 % with respect to albumin, in 0·01M phosphate buffer pH 6·8 and 0·2M NaCl. The runs were carried out as nearly as possible identically with respect to speed, temperature, etc. and photographs were taken at corresponding times, with identical settings of the optical system (Fig. 4). The mixture contains two main components: one, of $S^w_{20} = 3·57 \times 10^{-13}$, comprises 70 % of the total, has a sedimentation constant significantly lower than that of serum albumin alone ($4·11 \times 10^{-13}$) and the shape of the boundary suggests a more asymmetric particle; the other, of $S^w_{20} = 5·11 \times 10^{-13}$, comprises 30 % of the total and appears to be somewhat heterogeneous. There is no sign in the diagram of the mixture of any of the components of the polysaccharide, and the results provide clear evidence of complex formation be-

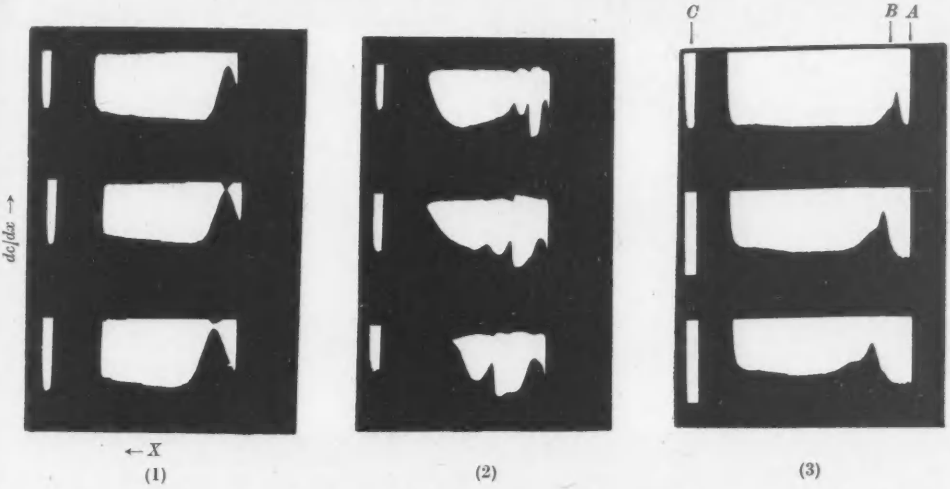

Fig. 3. (1) degraded polysaccharide: 1%, 20 min. intervals, 1080 r.p.s.; (2) undegraded polysaccharide: 0·5%, 10 min. intervals, 1000 r.p.s.; (3) antigen: 0·5%, 10 min. intervals, 370 r.p.s.

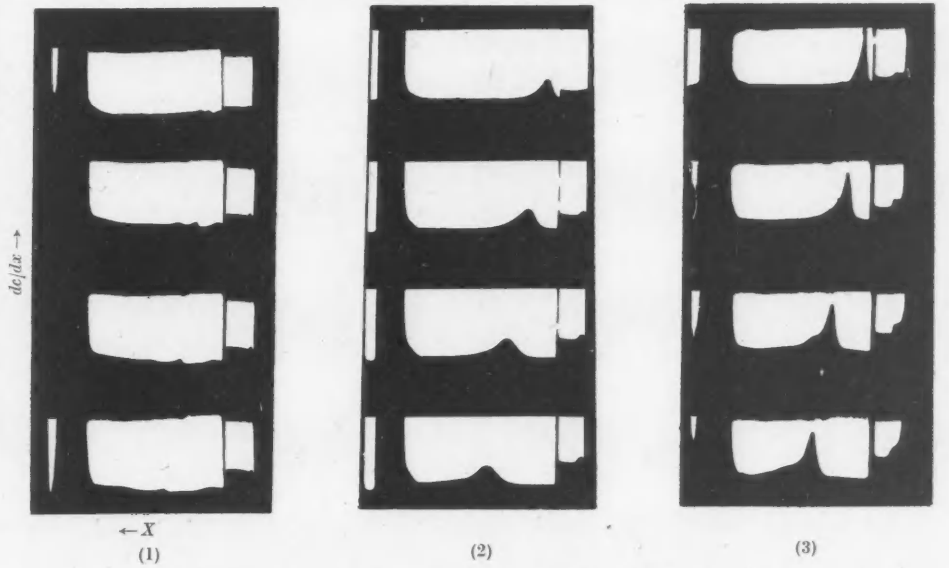

Fig. 4. (1) undegraded polysaccharide: 0·5%; (2) serum albumin: 1%; (3) undegraded polysaccharide: 0·5% + serum albumin, 1%, 20 min. intervals, 1050 r.p.s. Identical times and optical settings for all three sets of diagrams.

X = distance from centre of rotation; dc/dx = concentration gradient; A = meniscus; B = sedimentation boundary; C = index.

tween the protein and all components of the polysaccharide. This is in agreement with the titration data and the prevention of precipitation of the protein by trichloroacetic acid.*

resembles these rather closely, suggesting a similar type of combination with protein. Again, the main component (70 %) seems to be homogeneous, while the other (30 %) is rather heterogeneous. Only

Table 4. *Sedimentation constants of* Salmonella typhimurium *fractions*

Substance	Sedimentation constant ($S^{w}_{20} \times 10^{-13}$)	M (assumed value of V* in brackets)	Fraction of total refracting material	Specific refractive increment
Degraded polysaccharide	1·25	3,600 (0·62)	0·82	0·0012
Undegraded polysaccharide (0·5 %)	1·72	5,800 (0·62)	—	0·0012
	7·60	54,000 (0·62)		
	13·10	117,000 (0·62)		
Serum albumin	4·11	70,000	0·73	—
0·5 % undegraded polysaccharide + 1 % serum albumin	3·57	28,000 (0·71)	0·60	—
	5·11	48,000 (0·71)	0·23	
Antigen (0·526 %)	56·8	$1·5 \times 10^{6}$ (0·68)	0·31	0·00115
	79·7	$2·5 \times 10^{6}$ (0·68)	0·14	

* Partial specific volume.

(4) The bacterial antigen (Fig. 3) shows two sedimentation boundaries of $S^{w}_{20} = 79·7$ and $56·8 \times 10^{-13}$. Although the sedimentation rates are much greater than those of the polysaccharide-serum albumin mixture, the general character of the components

* The horse serum albumin was prepared from horse serum in 1938 by the method of Hewitt [1938] somewhat modified. It was free from carbohydrate and appeared to be homogeneous. It was lyophil dried. In the course of five years it seems to have become heterogeneous to some extent; the specific refractive increment in the boundary is only 0·00136 compared with 0·00185 when it was made. The sedimentation constant of the homogeneous part remains unchanged.

about 50 % of the total refracting material is present in these components. There is also a small amount of material which sediments very slowly.

The results are summarized in Table 4: column 2 gives the sedimentation constant in water at 20°; column 3 gives estimates of the molecular weights: it must be emphasized that these are calculated for spherical particles on the basis of the assumed values of the partial specific volumes given in brackets, and therefore do no more than indicate the orders of magnitude; column 4 gives the fraction of total refracting material appearing in the respective boundaries.

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The Reduction of Methaemoglobin by Ascorbic Acid

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In idiopathic methaemoglobinaemia, of which we have investigated some cases, the occurrence of an atypical form of methaemoglobin abnormally resistant to reduction has been postulated by Lian, Frumusan & Sassier [1939] as an aetiological factor. It thus became of interest to examine the evidence which has been put forward by Vestling [1942] against the unitary view of methaemoglobin. This author found that methaemoglobin made from haemoglobin by the action of nitrite was much less readily reduced by ascorbic acid than methaemoglobin formed by the action of ferricyanide, and suggested that these two spectroscopically similar products [Austin & Drabkin, 1935] differed chemically. The reaction is also of interest because it has been applied clinically by Deeny, Murdock & Rogan [1943] in the treatment of idiopathic methaemoglobinaemia.

The experiments reported here indicate that there is no chemical difference between nitrite- and ferricyanide-prepared methaemoglobin, so far as reduction by ascorbic acid is concerned, and that the differences observed by Vestling are due to the presence of ferrocyanide.

METHODS

Crystalline horse haemoglobin was prepared following Taylor & Hastings [1939], and was preserved in the ice-chest in contact with toluene. Methaemoglobin (MHb) was determined by the method of Evelyn & Malloy [1938], using a Hilger Spekker absorptiometer with filter no. 1 (red), of set H. 455, transmitting light of wave-lengths greater than 620 mμ. A calibration curve was prepared by using known amounts of MHb prepared from haemoglobin solutions which had been standardized with the Van Slyke-Neill manometric apparatus.

Determinations of total pigment were made by the method of Clegg & King [1942], again using the Spekker absorptiometer with filter no. 5 (green), of set H. 455 (maximal transmission between 500 and 580 mμ), and a calibration curve prepared with the aid of the Van Slyke apparatus.

In order to follow the course of the reaction between MHb and ascorbic acid, haemoglobin solutions having a concentration of about 10 g./100 ml. were prepared with M/20 phosphate buffer, pH 7·4, and partially converted to MHb by the addition of either one equivalent of $NaNO_2$ or of $K_3Fe(CN)_6$. The resulting solution (2 ml.) was placed in the main chambers of a series of Barcroft manometric flasks of the usual type. The required amount of *l*-ascorbic acid (B.D.H. synthetic), dissolved in M/3 phosphate buffer, pH 7·4 was contained in Keilin tubes. The manometers were filled with a mixture of 20% CO (prepared from formic acid), and 80% N_2. After equilibration by 15 min. shaking in the water-bath, the ascorbic acid was added from the Keilin tubes and the reaction followed by the uptake of CO by the haemoglobin formed from MHb. At the end of an experiment, the final concentration of MHb in each flask was estimated with the absorptiometer.

A few experiments were carried out in the presence of N_2 only; here a modified Thunberg tube was used with a tap attached to the bottom, by means of which samples of solution could be withdrawn during the course of an experiment. The MHb solution was placed in the main tube, the ascorbic acid in the side arm, and the vessel repeatedly evacuated and refilled with N_2. The solutions were then mixed and portions removed at intervals for determination of MHb.

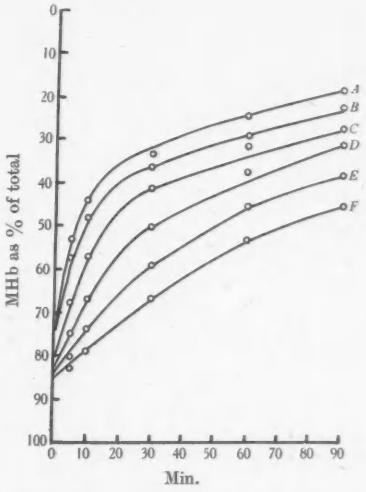

Fig. 1. The effect of ferrocyanide on the reduction of nitrite-methaemoglobin by ascorbic acid. In all manometers, 6·6 μmol. methaemoglobin and 6·6 μmol. ascorbic acid. $K_4Fe(CN)_6$: *A*, 13·2 μmol.; *B*, 6·6 μmol.; *C*, 3·3 μmol.; *D*, 1·7 μmol.; *E*, 0·8 μmol.; *F*, nil. Temp. = 28°. Total vol. in each, 2·2 ml.

RESULTS

The effect of ferrocyanide on the rate of reduction of nitrite-methaemoglobin. The effect of the addition of varying amounts of $K_4Fe(CN)_6$ on the reaction between nitrite-methaemoglobin (nitrite-MHb) and ascorbic acid is shown in Fig. 1. With the amount

of CO absorbed in the first 5 min. as a measure of the velocity of the reaction, it was found that the acceleration produced was directly proportional to the amount of ferrocyanide added until the level of one equivalent was reached; beyond this point the relation does not hold, in part because a 5 min. period is too long to give a true measure of the initial velocity of the reaction when this is high. It will be noticed that in Fig. 1 the amounts of MHb present initially are different. When ferrocyanide acts upon a solution containing MHb, the formation of haemoglobin and ferricyanide takes place until equilibrium is reached. Since the ferrocyanide was added to the MHb solutions before the beginning of the experiment, this reaction was largely completed during the equilibration period and led to a diminished initial MHb concentration in the manometers containing large amounts of ferrocyanide.

Comparison between ferricyanide-methaemoglobin and nitrite-methaemoglobin. The effect of the addition of ferrocyanide on the rate of reduction of nitrite-MHb made it likely that the observed difference between it and ferricyanide-methaemoglobin (ferricyanide-MHb) might be due to the

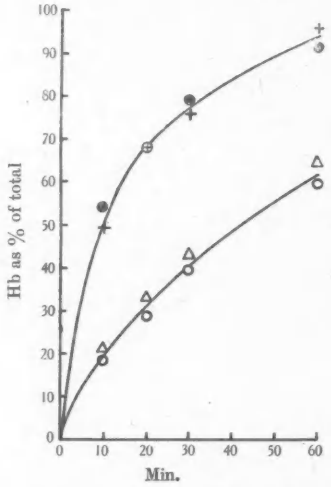

Fig. 2. Comparison between nitrite-methaemoglobin and ferricyanide-methaemoglobin. In each manometer 6·2 μmol. methaemoglobin and 6·2 μmol. ascorbic acid. Total volume 2·2 ml. Temp. = 37°. o Dialyzed ferricyanide-MHb. △ Nitrite-MHb. + Dialyzed ferricyanide-MHb + 6·2 μmol. $K_4Fe(CN)_6$. • Nitrite-MHb + 6·2 μmol. $K_4Fe(CN)_6$.

presence of ferrocyanide formed during the preparation of the latter. To test this point, a concentrated solution of ferricyanide-MHb was dialyzed in a collodion sac against tap water for 24 hr. The resulting solution had a MHb concentration of 5·2 g./100 ml.; the rate of reduction by two equivalents of ascorbic acid with and without one equivalent of potassium ferrocyanide was compared with the rate of reduction of a nitrite-MHb solution of equal concentration. The results are shown in Fig. 2 and support the view that there is no essential difference between the two types of MHb.

The mechanism of the catalytic action of ferrocyanide on the reduction of methaemoglobin. The most obvious suggestion is that the iron in the ferrocyanide acts as a catalyst by being alternately oxidized and reduced according to the following reactions:

$$Fe^{+++}\,(MHb) + \text{ferrocyanide} \rightarrow Fe^{++}\,(Hb) + \text{ferricyanide}.$$

$$\text{Ferricyanide} + \text{ascorbic acid} \rightarrow \text{ferrocyanide} + \text{dehydroascorbic acid}.$$

In order that the first reaction may proceed from left to right, it is a necessary condition that the ratio ferri-/ferro-cyanide be such that the e.m.f. of the system ($E_0' = +406$ mV. [Michaelis, 1933]), shall be less positive than that of the system MHb/Hb ($E_0' = +140$ mV. at pH 7 [Taylor & Hastings, 1939]). If equimolecular amounts of MHb and Hb were present, fulfilment of this condition would require that the ratio ferri-/ferro-cyanide should be less than 1:27,000. This consideration, however, does not rule out the possibility of this reaction in the experiments just quoted, because these were carried out in the presence of CO, which, by combining with free Hb, so alters the value of the quotient MHb/Hb that the effective E_0' of the system, as calculated from the equilibrium reached in the presence of ferrocyanide, becomes about +380 mV. It would be expected that in the absence of a gas with which haemoglobin combines, the catalytic effect of ferrocyanide would be much reduced or would disappear. Repetition of the experiments shown in Fig. 2 in an atmosphere of N_2 led to results which were, within experimental error, precisely similar to those obtained in the presence of CO, suggesting, therefore, that ferrocyanide, as such, does not act by being alternately oxidized and reduced. The true nature of the catalysis remains obscure.

Catalysis of the reduction of methaemoglobin by metals. The existence of ferrocyanide catalysis suggested that other substances might share this property and it has, in fact, been found that copper and iron both exert a considerable effect on the rate of reduction. The effect of $FeSO_4.(NH_4)_2SO_4.6H_2O$ and $CuSO_4$ is shown in Table 1. Some part of the 'normal' reaction between MHb and ascorbic acid is probably due to traces of metals present in the solutions, for it was found that saturation with 8-hydroxyquinoline (combining with copper) and $\alpha\alpha'$-dipyridyl (combining with ferrous iron) reduced the rate of reduction by some 50 and 20 % respectively.

Table 1. *Catalysis of the reduction of methaemoglobin by* Fe *and* Cu

	Metal catalyst (μg./ml.)	Methaemoglobin reduced in 30 min. (μmol.)
	None	3·4
Fe	25	5·1
	50	5·4
Cu	25	4·7
	50	5·1

Methaemoglobin (12·0μmol. in 2 ml.) + ascorbic acid (two equiv.). Vol. = 2·2 ml.; temp. = 28°. The data given are the means of duplicate determinations.

The mechanism of the reaction between nitrite-methaemoglobin and ascorbic acid. The reaction between MHb and ascorbic acid appears to be satisfactorily described by the equation for a bimolecular reaction, as shown by the reasonably constant values of k (velocity constant), obtained by substitution in the bimolecular formula. Table 2

Table 2. *Velocity constant for the reduction of methaemoglobin by ascorbic acid*

Time (hr.)	CO uptake (μl.)	k
0·17	14	1·43
0·50	34	1·33
1·0	50	1·15
1·5	63	1·13
2·0	73	1·15

Methaemoglobin (6·0μmol. in 2 ml.) + ascorbic acid (one equiv.). Vol. = 2·2 ml.; temp. = 37°.

shows the analysis of one experiment. Determination of the order of the reaction by observation of the time required to reach 50% reduction, starting with equivalent quantities of reactants at varying concentrations, also supported this conclusion (Table 3).

Table 3. *Inverse proportionality between concentration of reactants and time required to reach* 50% *reduction of methaemoglobin*

Initial [MHb] (g./100 ml.)	Time of 50% reduction (t, min.): Individual results	Mean	Product: [MHb] × t
10·0	50, 50	53	530
8·4	67, 66, 60	63	528
5·0	116, 102	109	545
4·2	116, 112, 108	112	470
		Av.	518

The effect of ascorbic acid concentration on the rate of reduction of methaemoglobin. The initial rate of reaction with constant MHb concentration should be proportional to the ascorbic acid concentration. An attempt to verify this relation was made by measuring the gas uptake during the first 10 min. of the reaction, with varied amounts of ascorbic acid in the presence of two equivalents of glutathione, which, while itself reacting only slowly with MHb, maintained the ascorbic acid in the reduced form and enabled measurable gas uptakes to be obtained with the smaller concentrations of ascorbic acid. The results are shown in Table 4. The apparent

Table 4. *Effects of ascorbic acid concentration and 8-hydroxyquinoline on reduction of methaemoglobin*

Ascorbic acid (μmol.)	μl. CO absorbed in 10 min.: 8-Hydroxyquinoline absent, Gross	8-Hydroxyquinoline absent, Net	8-Hydroxyquinoline present, Gross	8-Hydroxyquinoline present, Net	Inhibition (%)
—	11	—	10	—	—
0·8	31	20	16	6	70
1·5	43	32	20	10	69
3	54	43	25	15	65
6	73	62	36	26	58
12	89	78	45	35	55

Methaemoglobin (12μmol.) + glutathione (24μmol.). Vol. = 2·2 ml.; temp. = 37°.

velocity with small concentrations of ascorbic acid was much in excess of expectation, while the inhibition produced by saturation with 8-hydroxyquinoline was more marked in the case of low concentrations of ascorbic acid, suggesting that the reaction usually observed is made up of two components, the one a direct reaction between ascorbic acid and MHb, the other a reaction catalyzed by traces of metals in solution, the latter being little affected by increase of the ascorbic acid concentration beyond a low limiting value.

DISCUSSION

While similar behaviour towards ascorbic acid does not necessarily indicate that nitrite-MHb and ferricyanide-MHb are identical, the present results remove the experimental basis for the contrary view.

It may be remarked that Vestling [1942] found an equilibrium was reached between ferricyanide-MHb and two equivalents of ascorbic acid when 83–95% of the MHb had been reduced. In this series of experiments, it has been found that when sufficient time is allowed (24 hr. at room temperature) reduction proceeds so far that the residual MHb does not amount to more than 1–2% of the total pigment present. In view of the large difference between the potentials of the ascorbic acid and MHb/Hb systems in the presence of a gas combining with Hb, and the instability of dehydroascorbic acid, it would appear that the concept of an equilibrium must be largely illusory, and that Vestling's results may best be explained by the

occurrence of secondary oxidative changes (for his experiments were not carried out under anaerobic conditions), and by the probability that insufficient time was allowed for the reaction to go nearly to completion.

Extrapolation from the results obtained *in vitro* suggests that the rate of reduction of methaemoglobin, which could be expected with the concentrations of ascorbic acid likely to occur *in vivo*, would be small as compared with the rate of reduction of the pigment brought about by the enzymic mechanism of normal erythrocytes [Cox & Wendel, 1942]. The clinical use of ascorbic acid in the treatment of methaemoglobinaemia would thus appear to be limited to cases in which the normal enzymic mechanism for the removal of methaemoglobin is absent or has become suppressed.

SUMMARY

1. It has been confirmed that ferricyanide-methaemoglobin, as ordinarily prepared, is reduced more rapidly by ascorbic acid than is nitrite-methaemoglobin.

2. This difference is due to the catalytic effect of ferrocyanide on the reduction of methaemoglobin. There is thus no basis for the supposition that this effect indicates a chemical difference between the two forms of methaemoglobin.

3. Iron and copper salts produce similar catalytic effects.

4. The reaction between ascorbic acid and methaemoglobin is described by the equation for a bimolecular reaction.

The author wishes to thank Prof. D. C. Harrison for much helpful criticism and advice.

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The Assimilation of Glucose and Galactose in the Liver

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In this paper an attempt is made to examine the intermediary metabolism of glucose and galactose in the liver of the intact animal. *In vitro* experiments [Cori, Cori & Schmidt, 1939; Ostern, Herbert & Holmes, 1939] have proved that glucose-1-phosphate and hexose-6-phosphate are intermediaries in the glucose and glycogen metabolism of the liver. Both galactose-1-phosphate and glucose-1-phosphate have been found in the livers of rabbits assimilating galactose [Kosterlitz, 1937; 1943*a*]. In the present investigation, in addition to the phosphoric esters, glucose, galactose, and glycogen were estimated in the livers of rats at varying intervals after ingestion of glucose or galactose.

METHODS

General. Male rats from the Rowett Research Institute were kept on the stock diet. Prior to the experiment they were fasted for 24 hr., and then fed by stomach tube with either 1·5 ml. of 60% (w/v) galactose solution/100 g. body weight, or with 1·5 ml. of 55% (w/v) glucose solution. After 30, 60, 90 or 120 min. the rats were killed by stunning and the livers excised. After about 0·5 g. liver had been placed in 30% KOH for glycogen estimation, the remainder was frozen in liquid O_2; samples of the frozen liver were used for the various estimations.

Estimation of glucose and galactose. The $HgSO_4$-$BaCO_3$ method of West, Scharles & Peterson [1929] was used for the preparation of protein-free filtrates. Absence of traces of Ba^{++} and Hg^{++} was ensured by the addition of two drops of saturated solution of K_2SO_4 and of a small quantity of Zn powder. Glucose and galactose were estimated in the filtrate by the fermentation method previously described [Kosterlitz, 1937].

Estimation of glycogen. Our micro-adaptation of Pflüger's method was used [Kosterlitz, 1933], with 30% KOH as digestion fluid.

Estimation of reducing phosphoric esters, glucose-1-phosphate and galactose-1-phosphate. This method has previously been described [Kosterlitz & Ritchie, 1943]. The results are given under the following headings: (1) 'Initial reduction',

which is an approximate measure of the reducing phosphoric esters present. Since the nitrogenous esters have been removed, this fraction is composed mainly of equilibrium ester (glucose-6-phosphate and fructose-6-phosphate), possibly together with small quantities of pentosephosphates. If galactose-6-phosphate should be formed, it also would contribute to the 'initial reduction'; but so far there is no evidence that this ester is formed in the liver during galactose assimilation. (2) Increase in reducing power, calculated as glucose, after 3 min. hydrolysis at 100° ($Red._{100°}$) and after 30 min. hydrolysis at 50° ($Red._{50°}$), and increase in inorganic P (uncorrected) after 3 min. hydrolysis at 100° ($P_{100°}$) and 30 min. hydrolysis at 50° ($P_{50°}$). The values thus obtained are used for characterizing the 1-esters present: glucose-1-phosphate has $Red._{50°}/Red._{100°} = P_{50°}/P_{100°} = 0{\cdot}286$ and $Red._{100°}/P_{100°} = Red._{50°}/P_{50°} = 5{\cdot}81$, while galactose-1-phosphate has $Red._{50°}/Red._{100°} = 0{\cdot}775$, $P_{50°}/P_{100°} = 0{\cdot}765$, $Red._{100°}/P_{100°} = 4{\cdot}63$, and $Red._{50°}/P_{50°} = 4{\cdot}70$.

In experiments on the addition of 1-esters to CCl_3COOH extracts of liver only about 80% of the added material was recovered: in this paper no correction has been made for this loss of 20%.

Significance of results. The statistical methods described by Fisher [1941] were used.

found for $Red._{50°}/Red._{100°}$, $Red._{100°}/P_{100°}$, and particularly $Red._{50°}/P_{50°}$ suggested, however, that besides glucose-1-phosphate an unidentified substance was present, which was probably not a phosphoric ester. It was about as sensitive to acid hydrolysis as galactose-1-phosphate and there was no further increase in reducing power when the hydrolysis at 100° was extended from 3 to 20 min.

Some variation was found in the glycogen contents of the livers. There was, however, a remarkably close relationship between the glycogen and glucose contents, which varied in the same direction and were sometimes almost identical. This phenomenon was absent in rats fed either glucose or galactose. Similarly, Guest [1941] found in fasted but not in fed rats that the mean blood sugar concentrations were directly proportional to the logarithms of the liver glycogen concentrations.

Rats fed with glucose (Tables 1 and 2). The glycogen content of the liver increased with prolongation of the period of glucose absorption, but otherwise

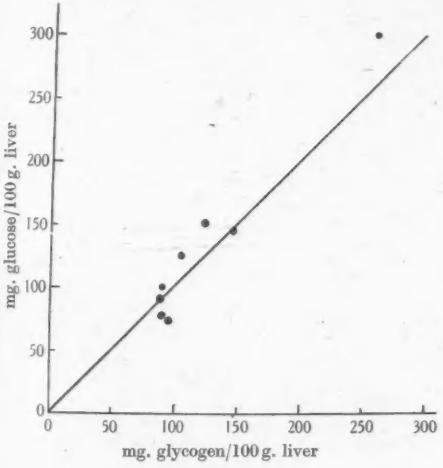

Fig. 1.

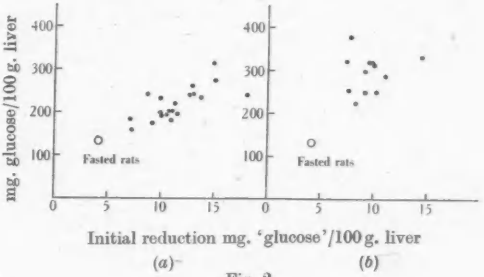

Fig. 2.

Fig. 1. Relationship between glucose and glycogen contents of livers of rats fasted for 24 hr.

Fig. 2. Correlation between glucose and reducing phosphoric ester levels in the livers of rats fed with (*a*) galactose, and (*b*) glucose. Correlation coefficients: (*a*) $r = +0{\cdot}6926$, $P < 0{\cdot}01$; (*b*) $r = +0{\cdot}1457$, $P > 0{\cdot}1$.

RESULTS

Rats fasted for 24 *hr.* (Fig. 1 and Table 2). Very small quantities of acid-labile phosphoric esters appear to be present in the liver. The ratio $P_{50°}/P_{100°}$ was 0·225, agreeing approximately with the value calculated for glucose-1-phosphate. The high values

the duration of the absorption had no influence on the results. For the purpose of comparing the reducing phosphoric ester and glucose-1-phosphate levels of the glucose-fed rats with those of the fasted ones, the results obtained after feeding glucose were pooled. There was a highly significant increase in both esters after glucose administration. On the

Table 1. *Data relating to carbohydrate and phosphoric ester contents of livers of rats given different sugars*

Sugar fed	Time elapsing between feeding of sugar and death of animal (min.)	No. of rats in group	Mean weight of group (g.)	Liver: Weight (g./100 g. body wt.)	Liver: Glycogen content (g./100 g.)	Liver: Glucose content (g./100 g.)	Liver: Galactose content (g./100 g.)	Liver: Initial reducing power (approx. measure of reducing, i.e. hexose-6-phosphoric esters) (mg. 'glucose'/100 g.)	Liver: Increase in reducing power or inorganic P content (approx. measure of hexose-1-phosphates) (mg./100 g.): After 3 min. hydrolysis at 100°: 'Glucose'	After 3 min. hydrolysis at 100°: Inorganic P	After 30 min. hydrolysis at 50°: 'Glucose'	After 30 min. hydrolysis at 50°: Inorganic P
Glucose	30	3	293 ±16	2·86 ±0·03	0·331 ±0·006	0·331 ±0·023	—	8·0 ±0·52	2·25 ±0·22	0·17 ±0·051	1·25 ±0·13	0·07 ±0·039
	60	3	278 ±21	2·85 ±0·05	0·741 ±0·102	0·284 ±0·017	—	9·5 ±1·02	2·7 ±0·09	0·185 ±0·044	1·4 ±0·14	0·045 ±0·035
	90	3	292 ±34	2·90 ±0·15	1·52 ±0·075	0·277 ±0·027	—	11·2 ±1·63	2·3 ±0·15	0·22 ±0·032	0·9 ±0·09	0·065 ±0·035
	120	3	280 ±25	3·11 ±0·09	2·06 ±0·173	0·285 ±0·031	—	9·15 ±0·48	2·65 ±0·12	0·245 ±0·018	1·5 ±0·24	0·065 ±0·023
Galactose	30	5	319 ± 9	2·75 ±0·06	0·259 ±0·047	0·218 ±0·028	0·181 ±0·013	10·5 ±1·61	4·35 ±0·26	0·56 ±0·036	2·7 ±0·28	0·315 ±0·010
	60	5	296 ±12	2·59 ±0·09	0·469 ±0·089	0·214 ±0·016	0·227 ±0·020	11·4 ±0·60	4·3 ±0·33	0·60 ±0·033	2·9 ±0·24	0·36 ±0·002
	90	5	301 ±19	2·70 ±0·07	0·719 ±0·112	0·239 ±0·011	0·281 ±0·026	11·5 ±1·13	5·0 ±0·15	0·57 ±0·048	3·05 ±0·17	0·34 ±0·020
	120	5	287 ±12	2·86 ±0·14	0·825 ±0·066	0·206 ±0·010	0·277 ±0·025	12·2 ±1·51	4·85 ±0·38	0·71 ±0·038	3·15 ±0·34	0·455 ±0·046

In the expression 293 ±16, 16 is the standard deviation of the mean.

Table 2. *Interpretation of carbohydrate and phosphoric ester analyses*

	Liver: Glucose (g./100 g.)	Liver: Hexose-6-phosphates. Initial reducing power (mg. 'glucose'/100 g.)	Liver: Hexose-1-phosphates: Increase in reducing power or inorganic P content (mg./100 g.): After 3 min. hydrolysis at 100°: 'Glucose' (=$Red._{100°}$)	After 3 min. hydrolysis at 100°: Inorganic P (=$P_{100°}$)	After 30 min. hydrolysis at 50°: 'Glucose' (=$Red._{50°}$)	After 30 min. hydrolysis at 50°: Inorganic P (=$P_{50°}$)	$Red._{50°}/Red._{100°}$	$P_{50°}/P_{100°}$	$Red._{100°}/P_{100°}$	$Red._{50°}/P_{50°}$
Mean of 10 fasted rats	0·133 ±0·026	4·13 ±0·40	1·54 ±0·09	0·089 ±0·009	0·84 ±0·09	0·020 ±0·007	0·545	0·225	17·3	42·0
Mean of 12 glucose-fed rats	0·294 ±0·012	9·45 ±0·56	2·48 ±0·09	0·205 ±0·019	1·28 ±0·10	0·060 ±0·015	0·516	0·293	12·1	21·3
Mean of 20 galactose-fed rats	0·219 ±0·009	11·4 ±0·60	4·63 ±0·15	0·610 ±0·023	2·94 ±0·13	0·367 ±0·017	0·635	0·602	7·6	8·0
Mean of glucose-fed minus mean of fasted rats	0·161	5·32	0·94	0·116	0·44	0·040*	0·468	0·345	8·1	11·0
Mean of galactose-fed minus mean of fasted rats	0·086	7·27	3·09	0·521	2·10	0·347	0·680	0·666	5·93	6·05
Mean of galactose-fed minus mean of glucose-fed rats	−0·075	1·95*	2·15	0·405	1·66	0·307	0·772	0·758	5·31	5·41
Calculated for: Glucose-1-phosphate							0·286	0·286	5·81	5·81
Galactose-1-phosphate							0·775	0·765	4·63	4·70

In the expression 0·133 ±0·026, 0·026 is the standard deviation of the mean.

* The differences of the means are all significant, the values marked by an asterisk having P between 0·05 and 0·02 and the others $P < 0·01$ [Fisher, 1941].

other hand, no correlation was found between liver glucose, glycogen, reducing phosphoric esters and glucose-1-phosphate. The ratios Red.$_{50°}$/Red.$_{100°}$ and Red./P were somewhat smaller than those in the fasted rats but still greater than the values calculated for glucose-1-phosphate. Similarly the values obtained from the differences of the means of the glucose-fed and fasted rats were still too high for glucose-1-phosphate. This may indicate an increase in the unknown acid-labile substance already found in the livers of fasted animals, but the number of observations is too small to make any definite statement on this point.

Rats fed with galactose (Tables 1 and 2; Fig. 2). There was a gradual increase in the liver galactose and glycogen with increased duration of galactose absorption, which had no other significant influence on the results, with the possible exception of the 120 min. P values. The glucose levels were lower than those in the rats fed glucose. The reducing phosphoric esters (pooled results on 20 rats) were slightly higher in the animals fed galactose than in those fed glucose. There was a highly significant positive correlation between glucose and reducing phosphoric esters which was absent in the glucose-fed rats (Fig. 2). The 1-phosphates were considerably higher than in the fasted and the glucose-fed animals. Both ratios, Red.$_{50°}$/Red.$_{100°}$ and $P_{50°}/P_{100°}$, were near 0·6, a value indicating a mixture of approximately 1 part glucose-1-phosphate (0·203 mg. P/100 g. liver) and 2 parts galactose-1-phosphate (0·406 mg. P/100 g. liver) [Kosterlitz & Ritchie, 1943]. The ratios Red./P were too high for either glucose-1-phosphate or galactose-1-phosphate. This possibly indicates an increase in the unknown acid-labile substance, already noted in the case of the glucose-fed animals. If the ratios were calculated from values obtained by deducting the means of the values for the fasted rats from those for the galactose-fed ones, a small increase of the ratios Red.$_{50°}$/Red.$_{100°}$ and $P_{50°}/P_{100°}$ was found, and a decrease of the Red./P ratios. The data thus obtained indicated a mixture of approximately 1 part glucose-1-phosphate (0·104 mg. P/100 g. liver) and 4 parts galactose-1-phosphate (0·416 mg. P/100 g. liver). From these calculations, and the values given for the glucose-fed rats (Table 2), one may conclude that in the galactose-fed rats 0·41 mg. galactose-1-phosphate P and 0·2 mg. glucose-1-phosphate P were present in 100 g. liver, and that in both glucose-fed and galactose-fed rats the same quantity of glucose-1-phosphate was formed, namely, that corresponding to 0·11 mg. P. This conclusion is further supported by the fact that the ratios calculated from the differences between the mean values for the galactose-fed and those for the glucose-fed rats were very close to the ratios calculated for galactose-1-phosphate: Red.$_{50°}$/Red.$_{100°}$ was 0·772 and $P_{50°}/P_{100°}$ 0·758 instead of 0·775 and 0·765 respectively, and Red.$_{100°}$/$P_{100°}$ 5·31 and Red.$_{50°}$/$P_{50°}$ 5·41 instead of 4·63 and 4·7 respectively; the P content was 0·405 mg./100 g. liver.

DISCUSSION

Rats assimilating glucose. Besides a deposition of glycogen and an increase in glucose, the administration of glucose caused an increase in the reducing phosphoric ester and glucose-1-phosphate levels of the liver. This is in agreement with the generally accepted theory that glycogen formation from glucose is preceded by the formation of glucose-6-phosphate and glucose-1-phosphate [Colowick & Sutherland, 1942]. Apart from glycogen, no metabolite accumulated in the liver during the assimilation of glucose.

Rats assimilating galactose. The deposition of glycogen was much smaller in the galactose-fed than in the glucose-fed rats, a fact already frequently observed [for literature see Deuel, 1936].

The glucose content of the liver was higher than in the fasted rats but lower than in the glucose-fed ones. The rise in liver glucose after galactose administration demonstrates the conversion of galactose to glucose. Harding, Grant & Glaister [1934] could find no significant increase in liver glucose after the administration of galactose, but the quantities given were much smaller than those used here.

Although the experimental evidence available is incomplete, it is improbable that the reducing phosphoric ester fraction isolated from the liver during galactose assimilation contains galactose-6-phosphate. This fraction has not yet been specially examined for the presence or absence of galactose-6-phosphate; however, prolonged acid hydrolysis did not liberate more galactose from the hexosemonophosphate fraction than a brief period of hydrolysis which is sufficient to hydrolyze galactose-1-phosphate but which hardly affects the difficultly hydrolyzable galactose-6-phosphate [Kosterlitz, 1937]. Further, Grant [1935] could not find any evidence for galactose-6-phosphate as an intermediary in the fermentation of galactose by galactose-adapted yeast.

The positive correlation between liver glucose and reducing phosphoric esters in the galactose-fed rats is remarkable, particularly as no such correlation was found in the glucose-fed animals. In the conversion of galactose to glucose, the reducing phosphoric esters may play an important role, glucose-6-phosphate being the most probable precursor of glucose.

Both galactose-1-phosphate and glucose-1-phosphate were found in the livers of rats assimilating galactose; this confirms previous findings on the rabbit [Kosterlitz, 1937; 1943*a*]. Similar quantities

The differences of the means are all significant, the values marked by an asterisk having P between 0·05 and 0·02 and the others $P<0·01$ [Fisher, 1941].

of glucose-1-phosphate were formed during galactose and glucose assimilation, corresponding to 0·11 mg. P or 0·6 mg. glucose/100 g. of liver. If the values for glucose-1-phosphate found during the assimilation of galactose by the rabbit [Kosterlitz, 1943*a*] are expressed in similar terms, a value of 0·16 mg. P/100 g. of liver is obtained. The galactose-1-phosphate content of the rat livers corresponded to 0·41 mg. P or 2·4 mg. galactose/100 g. and that of the rabbit livers to 0·9–2 mg. P or 5–12 mg. galactose/100 g. No explanation can be offered for the difference in reaction of rabbit and rat; the quantity of galactose-1-phosphate found in the liver is possibly related to the galactose tolerance of the species, which is very low in the rat. Kjerulf-Jensen [1942] determined galactose-1-phosphate in the livers of rats assimilating galactose; his values were much higher than those given in the present paper. His method of estimation, however, was not specific for hexose-1-phosphates; the acid-labile P was determined in the fraction of water-soluble Ba-salts isolated from the liver, without removal of the interfering acid-labile P compounds precipitable by Hg acetate [Kosterlitz & Ritchie, 1943]; there was no determination of the simultaneous increase in reducing power, and no estimation of the rate of acid hydrolysis.

No progressive accumulation of galactose-1-phosphate was found in the liver during galactose assimilation, a fact which makes it improbable that the rate of conversion of galactose-1-phosphate to a yet unknown metabolite is slower than that of galactose phosphorylation. Galactose-1-phosphate is very much more resistant to the enzymes present in liver slices, minced liver or aqueous liver extracts than glucose-1-phosphate [Kosterlitz & Ritchie, 1941]. Although the presence of galactose-1-phosphate, the increases in glucose-1-phosphate and reducing phosphoric ester levels, and particularly the correlation found to exist between reducing phosphoric esters and glucose after administration of galactose, suggest that all these substances take part in the conversion of galactose to glucose in the liver, the connecting link between the galactose and glucose compounds is still missing. In an attempt to formulate a tentative hypothesis of galactose fermentation [Kosterlitz, 1943*b*], galactose-1-phosphate was assumed to be converted to hexose-6-phosphate (Robison ester), probably by way of glucose-1-phosphate. This hypothesis was mainly based on the finding that galactose-1-phosphate and glucose-1-phosphate were fermented at almost identical rates by galactose-adapted yeast. Possibly a similar process takes place in the liver during galactose assimilation, the glucose phosphates then forming glycogen and glucose.

SUMMARY

1. The livers of fasting rats, and of those fed with glucose or with galactose, were analyzed for glucose, galactose, glycogen, glucose-1-phosphate, galactose-1-phosphate and reducing phosphoric ester (mainly glucose-6-phosphate and fructose-6-phosphate).

2. In the livers of fasting rats, reducing phosphoric esters and a small quantity of glucose-1-phosphate (0·09 mg. P/100 g.) were present. Further, there was evidence of the presence of a compound which was probably not a phosphoric ester and which yielded reducing substances on acid hydrolysis. In fasted rats, but not in fed ones, there was a very close direct relationship between the glycogen and glucose contents of the livers.

3. In the livers of rats fed with glucose there was an increase in the reducing phosphoric ester and glucose-1-phosphate (+0·11 mg. P/100 g.) levels.

4. During the assimilation of galactose the livers contained galactose-1-phosphate (0·41 mg. P/100 g.). Glucose-1-phosphate was present in quantities similar to those found after glucose administration, the ratio, glucose-1-phosphate formed to galactose-1-phosphate, being approximately 1/3·5. The livers contained less glucose and slightly more reducing phosphoric esters than those of glucose-fed rats. There was a high correlation between the glucose and reducing phosphoric ester levels, which was absent in glucose-fed animals. The significance of these findings for the mechanism of the conversion of galactose to glucose is discussed.

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The Assessment of Vitamin C Nutrition in Man

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As our knowledge of the part played by ascorbic acid in the maintenance of the intercellular architecture grows [Aschoff & Koch, 1919; Dalldorf, 1938] and in turn the importance of the latter in wound healing [Lund & Crandon, 1941; Bartlett, Jones & Ryan, 1942], the need for a reliable and simple assessment of the nutritional status with respect to this vitamin is obvious. A diminished supply of vitamin C-rich foodstuffs in the war-time diet of some adolescents and adults increases the urgency.

Two methods have been widely used: (1) the urinary saturation test, and (2) an examination of the concentration of ascorbic acid in whole blood, white cell-platelet layer or plasma.

Many of the conflicting reports in the literature concerning the urinary saturation test are undoubtedly attributable to the administration of widely different dosages of ascorbic acid ranging from 50 to 1000 mg./day, orally or intravenously in whole or divided doses and to differences in times of collection of urine. Data for normal daily urinary excretion of ascorbic acid vary from zero [Roe & Hall, 1939] to 40 mg. [Portnoy & Wilkinson, 1938].

Whole-blood determinations present some technical difficulties which are not easily offset. Haemolysis causes oxidation of ascorbic acid and inhibition of this by saturation with CO leads to indefiniteness in the end-point of the titration. Determination of the ascorbic acid content of the white cell-platelet layer involves a more complex technique and requires special apparatus which most laboratories would not possess.

It would be of great advantage to replace the somewhat laborious and unsatisfactory technique of the urinary saturation test, even in its most abbreviated form [Harris & Abbasy, 1937], by one determination. We have therefore endeavoured to establish whether a single determination of the plasma ascorbic acid concentration could be used to assay reliably the status of vitamin C nutrition of a subject. Opinion as to the value of isolated determinations of the plasma ascorbic acid content is equally divided; Portnoy & Wilkinson [1938], Ralli & Sherry [1941], and others hold the view that it is of value. The first named say that it may prove to be better than 'saturation' tests, whilst Kellie & Zilva [1939], Storvick & Hauck [1942], among others, hold the opposite view. Our results indicate that the plasma ascorbic acid determination can be used to assay the standard of vitamin C nutrition, in contrast to the unreliability of observations of the so-called 'resting' urinary daily output.

The work here reported on is compiled from data obtained from 50 subjects and falls into two categories: (*a*) the correspondence between the results of urinary 'saturation' tests and those of the determination of plasma ascorbic acid content, and (*b*) the assessment of the nutritional status of the subject with respect to ascorbic acid.

METHODS

Plasma ascorbic acid. 10 ml. of blood were run into a tube containing 0·1 ml. 20% potassium oxalate and 0·05 ml. 5% KCN, and the whole was mixed and immediately centrifuged. To 4 ml. of the plasma, which should show no haemolysis, were added 16 ml. of freshly prepared 3% HPO_3 in glass-distilled water. After centrifugation, 5 ml. portions were titrated with 0·01% 2:6-dichlorophenolindophenol. No difficulty was experienced in determining a stable end-point if the titration was carried out rapidly, and the results were highly reproducible by both of us. Visual titration was found to be as accurate as photoelectric methods [cf. Mindlin & Butler, 1937–8].

Pijoan & Klemperer [1937] added KCN (5 mg.) to inhibit oxidation of ascorbic acid in freshly drawn venous blood. Barron, Barron & Klemperer [1936] considered that addition of KCN may enhance the ascorbic acid value. Golden & Garfinkel [1942], who added ascorbic acid to the plasma, confirmed Borsook, Davenport, Jeffreys & Warner [1936–7] and Kassan & Roe [1940] in finding that ascorbic acid was preserved best in whole blood, especially at low temperatures of the order of 5–8°, whereas once the plasma had been separated little loss occurred in the first 3 hr. at these temperatures, though there was a rapid loss at room temperature. Our experiments on blood containing a high, but physiological, concentration of ascorbic acid, indicate that KCN does not enhance the reduction titre under the conditions employed, but stabilizes the plasma value.

Urine ascorbic acid and dehydroascorbic acid. Urine was collected in dark-coloured Winchesters containing 70 g. HPO_3. 0·05 ml. 0·10% 2:6-dichlorophenolindophenol was titrated with the urine, the latter diluted with a suitable volume of 3% HPO_3 if necessary [cf. Harris & Ray, 1935].

For dehydroascorbic acid a convenient volume of urine was treated with H_2S for 30 min. and then with N_2 for 15 min. after all traces of H_2S had been removed as shown by lead acetate paper. The resulting solution was used to titrate the dye.

The dye solutions used for blood and urine determinations were found to be stable for several weeks in the refrigerator if made in freshly boiled and cooled glass-distilled water. They were standardized in the usual way against ascorbic acid which in turn was standardized with $N/10$ I_2 solution.

Dosage and blood sampling. Unless stated to the contrary, a standard dose of 700 mg. of ascorbic acid/140 lb. body weight was given orally each day. The dose, in 50 mg. tablets, was administered as nearly as possible in two equal portions, one at the commencement of each 24 hr. collection of urine, the other approximately 12 hr. later. Blood samples were taken, either at the end of the 24 hr. period before the first dose of the subsequent 24 hr., or, if no dose was given, 3 hr. after taking food.

Todhunter, Robbins & McIntosh [1942] showed that after ingestion of synthetic ascorbic acid a maximum rise occurs in the plasma within 1–1½ hr., which may be delayed until 2½ hr. if a natural source of the vitamin, such as cauliflower, is employed. Initially high plasma levels (0·9 mg./100 ml.) may be raised above the level for the renal threshold by a 400 mg. test dose. The effective rise in the plasma level is correspondingly less if a smaller dose is ingested, or the initial level is appreciably lower. These findings justify our division of the standard dose of 700 mg./140 lb. body weight into two approximately equal parts at 12-hourly intervals in order to reduce the sudden overflow through the kidney to a minimum, an event which is more likely to occur the more nearly 'saturated' the subject.

Belser, Hauck & Storvick [1939] and Fincke & Landquist [1942] employed a test dose of 400 mg. and Goldsmith & Ellinger [1939] 300 mg. In general, a 50% excretion of the test dose has been taken as evidence of 'saturation'. Harris took as his criterion the first sharp rise in the urinary excretion, but later modified this to the excretion of 50 mg. or more in the period of 3 hr. commencing 4 hr. after the administration of the test dose. Wright, Lilienfeld & MacLenathen [1937], to avoid disturbance due to faulty absorption, employed a dose of 1000 mg. intravenously, 'saturated' subjects excreting more than 50% in the subsequent 5 hr.

RESULTS

(*a*) *Urinary and plasma ascorbic acid contents during extended saturation tests.* Investigations were carried out on nine subjects to study the behaviour of the plasma ascorbic acid during extended saturation of the subject with ascorbic acid. All subjects took, in addition to the test dose, the normal hospital diet. Evidence is presented elsewhere [Prunty & Vass, 1943] that such a diet is poor in vitamin C. It was thought necessary to establish the fact that the plasma ascorbic acid does not reflect the violent fluctuations which are well known to occur in the urinary output under these conditions, and therefore accurately reflects the intake of ascorbic acid at these levels.

Data typical of our results are given in Table 1 and Fig. 1. The latter brings out the point that the plasma ascorbic acid does in fact remain constant during prolonged saturation, very marked fluctuations in the urinary excretion being shown. Table 1 also indicates the importance of another fact in the interpretation of saturation. During the first period of saturation, 8–16 June 1942 (saturation being demonstrable on the latter date), the rise in the daily urinary excretion was relatively slow and no point of sharp demarcation was obvious. In this case the plasma ascorbic acid at the beginning of the test was 0·00 mg./100 ml., and such a low initial level tended to be associated with this phenomenon in other cases. A second saturation test carried out on the same subject with the plasma level initially at 0·26 mg./100 ml. shows on 15 August 1942 a spectacular rise in the excretion of ascorbic acid, an event easy of interpretation. Furthermore, on attaining saturation some of our subjects did not excrete as much as 50% of the test dose at any time. Therefore we have arbitrarily defined 'saturation' as the excretion of 33⅓% of the test dose based on 700 mg./140 lb. body weight, administered as outlined above.

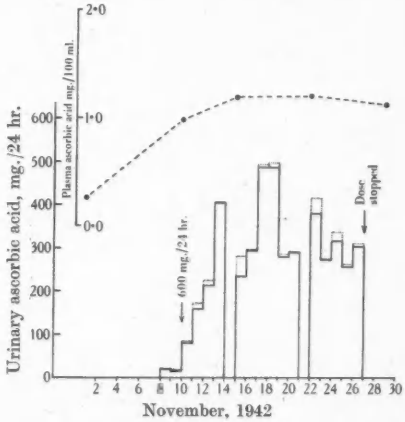

Fig. 1. Saturation test in subject J. Female. Abdominal sinuses. •-------• Plasma ascorbic acid, mg./100 ml. ——— Urine ascorbic acid, mg./24 hr. ------- Urine ascorbic acid + dehydroascorbic acid, mg./24 hr.

The data given in Fig. 1 serve to confirm the fact that the fluctuations in the urinary excretion of ascorbic acid during extended saturation are not due to compensating excretions of dehydroascorbic acid.

(*b*) *The relation of the plasma ascorbic acid to the 'state of saturation' of the subject and resting urinary excretion of 'ascorbic acid'.* The data on this point are compiled in part from that given in the last section and in part from additional data of which Fig. 2 is illustrative. It shows the usual saturation test carried out in six normal subjects with initial plasma levels ranging from 0·31 to 1·46 mg./100 ml. The striking correlation between the time of demonstration of saturation and the initial plasma level is

Table 1. *Extended ascorbic acid saturation test*

Subject B. Male. Gastric ulcer, partial gastrectomy. Body wt. 124 lb.

	Ascorbic acid					
Date	Dose (mg./day)	Urinary excretion (mg./day)	Percentage of dose excreted	Plasma conc. (mg./100 ml.)	Average urinary conc. over 24 hr. (mg./100 ml.)	Remarks
5. vi. 42	—	—	—	0·00	—	Confined to bed
6. vi. 42	—	16	—	—	0·64	Medical treatment for gastric ulcer
8. vi. 42	600	12	2	—	0·45	
9. vi. 42	600	11	2	0·55	0·50	
10. vi. 42	600	18	3	—	0·73	
11. vi. 42	600	17	3	—	0·65	
12. vi. 42	600	85	14	—	3·68	
13. vi. 42	600	118	20	1·54	3·51	
14. vi. 42	600	—	—	—	—	
15. vi. 42	600	186	31	—	3·36	
16. vi. 42	600	248	41	—	11·42	
17. vi. 42	600	268	45	—	10·05	
18. vi. 42	600	356	60	—	11·18	
19. vi. 42	600	269	45	1·99	11·00	
20. vi. 42	600	329	55	—	19·40	
21. vi. 42	600	—	—	—	—	
22. vi. 42	600	244	41	—	9·20	
23. vi. 42	600	348	58	—	17·60	
24. vi. 42	600	188	31	1·94	15·20	
25. vi. 42	600	267	45	—	11·70	
26. vi. 42	600	244	41	—	11·15	
27. vi. 42	600	257	43	—	11·36	
28. vi. 42	300	—	—	—	—	
29. vi. 42	300	103	34	—	5·64	
30. vi. 42	300	186	62	1·36	10·00	
1. vii. 42	300	88	29	—	5·16	
2. vii. 42	300	129	43	—	6·90	
3. vii. 42	300	134	45	—	8·55	
4. vii. 42	300	48	16	—	2·35	Got up
5. vii. 42	300	—	—	—	—	
6. vii. 42	300	106	35	1·29	5·76	
7. vii. 42	300	95	32	—	11·06	
8. vii. 42 to 17. vii. 42	50 to 50	—	—	—	—	
17. vii. 42 to 23. vii. 42	50 to 50	—	—	1·26	—	
23. vii. 42	50	18	36	—	0·93	
24. vii. 42	50	15	30	—	0·60	
25. vii. 42	50	12	24	0·90	0·62	
27. vii. 42	—	—	—	—	—	Operation
30. vii. 42 to 10. viii. 42	50 to 50	20	40	—	0·86	
10. viii. 42	50	10	20	—	0·66	
11. viii. 42	50	10	20	0·26	0·90	
12. viii. 42	50	18	3	—	0·88	
13. viii. 42	600	33	6	—	2·10	
14. viii. 42	600	70	12	—	5·73	
15. viii. 42	600	421	70	—	23·10	
16. viii. 42	600	460	77	—	32·60	
17. viii. 42 to 6. x. 42	600 to 100	—	—	—	—	Home
	100	—	—	0·62	—	

self-evident. Subject L., with a plasma ascorbic acid of 0·31 mg./100 ml., required 2 days to become saturated, whilst subject V. showed this in one day, the plasma level being initially 0·80 mg./100 ml.

Initial plasma ascorbic acid levels and the corresponding urinary ascorbic acid excretions before saturation with large doses of ascorbic acid, the dosage required to bring to a 'state of saturation', and the corresponding days required to demonstrate saturation are given in Table 2.

Fig. 3 shows the dose required to bring the subject to a 'state of saturation', and the initial plasma level. This dose was computed by determining the doses necessary to produce an excretion of one-third of the test dose (700 mg./140 lb. body weight) in 24 hr. If on a given day the excretion

was less than one-third and on the succeeding day the excretion was greater than one-third, the figure was obtained by interpolation. *From the total dose ordinate therefore represents the plasma concentration at the 'saturated state' corresponding to 0·8 mg./100 ml. Thus if the plasma ascorbic acid concentration of the subject, whose saturation is being investigated, is 0·8 mg./100 ml., administration of the test dose results in the excretion of one-third of the test dose. The plasma level reaches or even exceeds the renal threshold, placed by Ralli, Friedman & Sherry [1939] at 1·2–1·4 mg./100 ml.

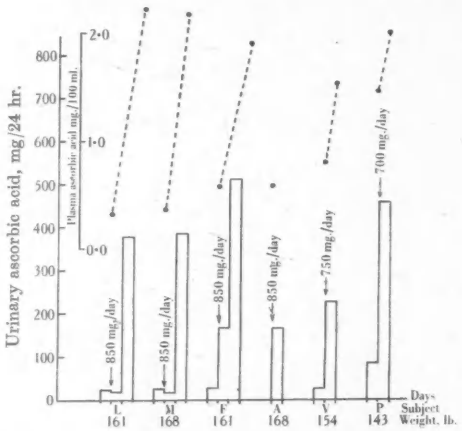

Fig. 2. Plasma level of six normal subjects before and after urinary saturation test. •-------• Plasma ascorbic acid, mg./100 ml. —— Urinary ascorbic acid, mg./24 hr.

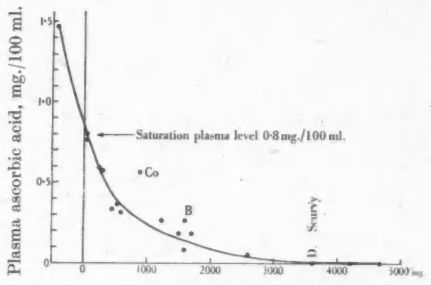

Fig. 3. Initial plasma ascorbic acid concentration against dose to bring to saturation level, with large doses. (700 mg./140 lb. body wt.)

are deducted 700 mg., which represent the single test dose per 140 lb. body weight necessary to demonstrate 'saturation' in a subject who has reached a 'state of saturation'. The point at which the curve crosses the

Table 2. *Initial plasma concentration and daily urinary excretion of ascorbic acid compared with total dosage mg./140 lb. body weight, and number of days required to bring subjects to a state of saturation*

	Ascorbic acid			
Subject	Initial plasma conc. (mg./100 ml.)	Initial urinary excretion (mg./day)	Dosage (mg.) required to bring to saturation level/140 lb. body wt.	Days to saturate
S.	0·0	50	4680	8
B.	0·0	24	4200	8
*D.	0·0	18	3620	6
Da.	0·05	24	2600	5
W.	0·08	31	1580	4
Ch.	0·18	13	1700	6
Sa.	0·18	18	1500	4
B.	0·28	13	1580	4
J.	0·26	17	1230	3
L.	0·31	26	580	2
C.	0·33	29	440	2
M.	0·36	24	520	2
Co.	0·56	7	870	3
F.	0·57	27	300	2
A.	0·59	—	230	2
P.	0·76	—	40	1
V.	0·80	27	40	1
Pr.	1·46	85	−430	1

* A case of clinical scurvy.

The curve indicates that the relationship between the two variables is not linear, a point that may partially account for the divergence of opinion in the literature as to the validity of 'resting' plasma ascorbic acid concentration as an index of vitamin C nutrition. It must be noted that the only marked deviation from the curve is that of case Co., a child aged 7 years, whereas the other points given are all for adults. The point B. was obtained in the second saturation test (Table 1). The point D. relates to a case of scurvy [Prunty & Vass, 1943].

In spite of the presence of unknown interfering substances, several authorities regard the titre of the 'resting urine' as significant an index as the 'saturation' test itself [Harris, Abbasy, Yudkin & Kelly, 1936; Portnoy & Wilkinson, 1938]. The former stated that 'as under ordinary circumstances the response to a test dose is proportional to the resting level, a measurement of the latter alone is sufficient for most routine tests of the kind here described'. If in an adult 13·8 mg. ascorbic acid are excreted each day, a good response to the test dose of 700 mg./140 lb. body weight is obtained on the first or second day. Table 2 shows that the 'resting' urinary excretion affords little evidence of the status of the vitamin C nutrition of any subject. D., who was clinically frankly scorbutic, excreted as much as the equivalent of 18 mg./day of ascorbic acid, whilst S. and B., who showed no such signs,

both requiring at least 6 days to become saturated, excreted much more than this in the 'resting urine' (see Table 2).

(c) *Comparison of the ascorbic acid intake with the maintenance of a known plasma ascorbic acid concentration.* During the course of this work it was possible to maintain only relatively few subjects on controlled intakes of ascorbic acid for prolonged periods during which observations of the plasma ascorbic acid concentration were made. A summary of the results obtained is presented in Table 3. In many cases the greater part of the ascorbic acid ingested was the synthetic product; the remainder of the intake was estimated from a dietetic history [cf. Harris & Olliver, 1943]. The figures fall into two groups, those obtained when the plasma level prior to the period of known intake was lower than the final level and vice versa.

shows that these fluctuations are not due to any interchange between these substances, which is in agreement with the findings of Johnson & Zilva [1934]. Examination of the two saturation tests in subject B. (Table 1) shows the differing urinary excretion responses which are met with in markedly unsaturated subjects (saturation not reached until the sixth day or later) compared with less unsaturated subjects (saturation reached by the fourth day) and illustrates the difficulty, in the earliest urinary saturation test, of noting the first sharp urinary response as an index of the achievement of saturation.

The data given in Table 2 and Fig. 3 establish the fact that the initial plasma ascorbic acid concentration is related to the dose of ascorbic acid required to bring the body to a 'state of saturation', which is attained when the plasma ascorbic acid

Table 3. *The relation between ascorbic acid ingested and the plasma ascorbic acid concentration*

	Ascorbic acid						
Subject	Initial plasma conc. (mg./100 ml.)	Daily dose (mg.)	Estimated dietary intake (mg./day)	Total daily intake (mg.)	Final plasma conc. (mg./100 ml.)	Duration of exp. (days)	Remarks
			A. Plasma concentration previously lower				
P.	0·15	300	50	350	1·52	88	
Ja.	—	150	70	220	1·26	1000	
De.	0·67	200	20	220	1·02	6	Received 75 mg./day for 14 days, 3 weeks prior to this
De.	—	100	20	120	0·67	7	
C.	—	50	50	100	0·33	7	
J.	—	—	70	70	0·26	28	Two oranges daily
			B. Plasma concentration previously higher				
B.	—	100	50	150	0·62	52	Saturated 52 days before
B.	0·90	50	20	70	0·26	38	Dose omitted for 3 days after partial gastrectomy, 3 days after plasma value was 0·90 mg./100 ml.
B.	1·29	50	20	70	1·26	9	
B.	1·26	50	20	70	0·90	21	
Ch.	2·17	50	20	70	1·31	8	Saturated 8 days before
Da.	1·24	50	20	70	1·06	6	Saturated 6 days before
Pr.	1·52	50	100	150	1·46	17	
Pr.	—	—	75	75	0·86	26	Saturated 26 days before
Pr.	0·86	—	50	50	0·58	23	
Pr.	0·58	—	50	50	0·41	23	

DISCUSSION

Our results show that the marked urinary fluctuations met with in the 'saturation' test are not mirrored in the plasma level. This supports the idea that the 'saturated state' is maintained and emphasizes the reliability of the plasma ascorbic acid content as an index of this state. Indeed, Harris [1942], in answer to his critics, considers that these fluctuations do not in any way invalidate the saturation test. An examination of the urinary excretion of dehydroascorbic acid and ascorbic acid

reaches 0·8 mg./100 ml. Harris [1942] stated that the necessity of maintaining a state of saturation for optimum nutrition is not established. Ralli & Sherry [1941, p. 290] maintained that in a 'normal' adult the optimal requirement of vitamin C was 100 mg. daily, 'this being the smallest daily dose on which saturation of the body tissues was attained and maintained'. These authors have endeavoured to assess the standard of nutrition in regard to vitamin C by examining the urinary excretion after administration of 100 mg. of the vitamin intravenously. Although they state that in normal (i.e. presumably saturated) subjects 50 % of the injected

vitamin is excreted within 3 hr., 8 out of 12 of their normal subjects failed to satisfy this criterion.

It is widely accepted that the dosage of ascorbic acid required to saturate the subject is a measure of the depletion of the available vitamin C reserves of the body, even though these may be small. It therefore follows from Fig. 3 that the lower the initial plasma ascorbic acid concentration the greater the degree of depletion of the available reserves of the body.

A summary of some of the 'normal' values for the ascorbic acid content of plasma which are to be found in the literature is tabulated below; this range is so great that it is of little clinical value.

Normal plasma values of ascorbic acid, mg./100 ml.

Secher [1942]	Down to 0
Wright, Lilienfeld & MacLenathen [1937]	0·27–1·54
Croft & Snorf [1939]	0·4
Ingalls [1937]	0·5 –2·0
Butler & Cushman [1940]	0·6
Portnoy & Wilkinson [1938]	0·6 –1·85
Abt & Farmer [1938]	0·7 –2·0
Fincke & Landquist [1942]	0·8
Abt, Farmer & Epstein [1936]	0·8 upwards

It is obvious from Fig. 3 that a plasma ascorbic acid value greater than 0·8 mg./100 ml. denotes an intake of ascorbic acid more than sufficient to satisfy the body's requirements and that at values at or near zero the ascorbic acid intake is grossly inadequate. This is borne out by the fact that scurvy does occur when the dosage requirement to bring to saturation is less than that of non-scorbutic subjects (see D., Fig. 3).

Examination of this curve reveals that the maximum change in the dosage of ascorbic acid required to produce a 'state of saturation' at a given plasma level occurs at a value slightly less than 0·4 mg./100 ml. plasma. In view of these facts it is clear that a plasma value of not less than 0·4 mg./100 ml. is compatible with a desirable standard of nutrition with respect to the vitamin. When the initial plasma ascorbic acid concentration is 0·4 mg./100 ml., 450 mg. of ascorbic acid per 140 lb. of body weight will bring the subject to a 'state of saturation' which can be demonstrated by the ingestion of a further 700 mg. of ascorbic acid. This is equivalent to a good saturation response in 2 days with the urinary saturation test of Harris [1943], who states that 'the number of days beyond the second may be taken as an index of the relative deficit in the past intake'.

The data presented in Table 3, although deficient in evidence relating to plasma ascorbic acid levels below 0·26 mg./100 ml., explain certain discrepancies met in the literature. Whereas Bryan, Turner, Huenemann & Lotwin [1941] and Ralli & Sherry [1941] found that a daily intake of 100 mg. of ascorbic acid stabilized the plasma level at approximately 1 mg./100 ml., our results suggest that a daily intake of at least 100 mg. is required to attain a 'state of saturation' with a plasma ascorbic acid content of 0·8 mg./100 ml. Our results also show that if the subject has previously been on a higher intake of ascorbic acid and reverts to a lower one, the plasma ascorbic acid tends to remain at a higher level than that reached when the procedure is reversed, unless a considerable period on the lower intake is maintained (probably at least 30 days). For example, after 21 days on an intake of 70 mg. ascorbic acid daily (subject B), where the initial plasma level was 1·26 mg./100 ml., the plasma level only fell to 0·90 mg./100 ml. This intake, continued for a total of 38 days, gave a final plasma level of 0·26 mg./100 ml. Subject J., on the other hand, who had been receiving the equivalent of 70 mg. daily, had a plasma content of 0·26 mg./100 ml. after 28 days, the dietetic history indicating that the initial plasma level was below that at the end of the test period.

Göthlin [1934], on the basis of his capillary fragility test, suggested at least 19–27 mg. of ascorbic acid daily were necessary to prevent scurvy in the adult. Fox, Dangerfield, Gottlich, Jokl & Suzman [1940] showed that a daily intake of 15–25 mg. of ascorbic acid did not prevent scurvy in a group of Witwatersrand miners. By contrast, Kellie & Zilva [1939] estimated that daily doses of 15 mg. ascorbic acid would suffice to prevent scurvy and maintain good health. They endeavoured to ascertain the minimum daily dose required to maintain 'saturation' and considered this to be achieved when, on a given dose, the ascorbic acid voided in the urine became constant from day to day. Having regard to the overlapping of an increased or decreased daily excretion as the dosage was stepped up or down, they studied the level of excretion reached on ascending from a state of 'unsaturation' and vice versa, with daily doses of 30, 50 and 100 mg. As a result they stated that 'it may be assumed that the minimum dose necessary to bring about "saturation" lies between 30 and 50 mg./day, probably nearer the former figure'. Their interpretation of the results obtained on A.E.K. do not agree with our results on subject B., Table 1, who on 23–25 July 1942 inclusive was excreting diminishing amounts of ascorbic acid in the urine in spite of the fact that the plasma level at the time was 0·9 mg./100 ml. and that B. had been receiving at least 50 mg. ascorbic acid per day for 16 days following a period of 'saturation'. Moreover, the high plasma levels observed in A.E.K. on a continually falling dose agree with our findings in Table 3. Ralli & Sherry [1941] have determined the plasma ascorbic acid values when the subject was on a known intake of vitamin C. With a daily intake of 50 mg., the plasma concentration varied from 0·28 to 0·30 mg./100 ml. when the initial and final plasma concentrations were not appreciably different. When the initial values were 0·97 and 1·45 mg./100 ml. the final values were 0·80 and 0·60 mg./100 ml. (cf. Pr., Table 3, initial value 0·86 mg./100 ml., daily dose 50 mg., value in 23 days 0·58 mg./100 ml., after 46 days 0·41 mg./100 ml.).

On 75 mg. daily intake, when the initial values were 0·3, the final values were 0·5 and 0·7 mg./100 ml.; when the initial value was 1·10 mg./100 ml., the final value was 0·8 mg./100 ml. On 100 mg. daily intake the initial values given are 0·2, 0·5, 0·7, 0·8, 1·25 and the respective final values, 1·07, 1·2, 1·2, 1·12, 1·05 mg./100 ml. These authors computed the daily requirement for an optimal nutrition at 100 mg. of ascorbic acid per day in the adult, by determining the amount retained on a known daily intake, a hazardous procedure in our opinion, in view of the marked

urinary fluctuations they also observed and by reason of the fact that it is not yet possible to determine quantitatively all the metabolic products of ascorbic acid, other than dehydroascorbic acid, which may be eliminated in the urine. Their results also show, as we have observed, that during prolonged saturation the urinary ascorbic acid content tends to fall to a lower value than that initially obtained, which is a further possible source of error in the computation of the ascorbic acid retention.

The most widely accepted figures of the daily requirements for vitamin C in this country and in America are respectively those of The Technical Commission of The League of Nations, Geneva [Bigwood, 1939] (30 mg./day) and The Committee on Food and Nutrition, National Research Council (U.S.A.) [1941] (75 mg./day). Whilst our evidence presented in Table 3 is insufficient a basis on which to dogmatize, it does suggest that at least 70 mg. of ascorbic acid daily are required for a plasma level of the order of 0·4 mg./100 ml. This conclusion, with that of Ralli & Sherry [1941] quoted above, together with the confirmatory observations of Bryan *et al.* [1941] that an intake of approximately 70 mg./day maintained a plasma level of 0·6 mg./100 ml., suggests that at least 60 mg./day are required to maintain a plasma level of as much as 0·4 mg./100 ml. This is in agreement with the view of Bacharach & Drummond [1940] and the Committee on Food and Nutrition (U.S.A.) quoted above, that the requirement of ascorbic acid is 75 mg./day, and that a well-balanced diet should contain this amount.

SUMMARY

1. The plasma ascorbic acid concentration is in man an index of the nutritional state with respect to vitamin C, unless, within the previous 30 days, the subject has been on an appreciably higher intake of ascorbic acid than he is receiving at the time of the test. For clinical purposes this determination is more easily carried out than a urinary saturation test.

2. A plasma ascorbic acid level of 0·8 mg./100 ml. represents a 'state of saturation'.

3. Evidence is presented for the desirability of a plasma ascorbic acid concentration of not less than 0·4 mg./100 ml.

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A Critical Examination of Lugg's Method for the Determination of *l*-Ascorbic Acid

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Recently Lugg [1942 *a*, *b*] has described a procedure designed to increase the specificity of the determination of *l*-ascorbic acid by indophenol titration. The method is based on the differential rates of combination of *l*-ascorbic acid and some other indophenol-reducing substances with HCHO at pH 1·5 and at 3·5. After reaction with HCHO these substances no longer reduce the indicator. In order to ascertain the limitations of this method, Lugg's results have been submitted to a critical examination. In addition we record very briefly some experiments from an investigation which was in progress when Lugg's paper appeared. These were also intended to serve as a basis for a more specific determination of *l*-ascorbic acid and were concerned with the differential properties of the primary oxidation products of *l*-ascorbic acid, reductone and reductic acid. Although the results obtained were less satisfactory than Lugg's, they were not without certain interest.

METHODS

In our investigation we have introduced a few minor modifications which in no way interfered with the general principle enunciated by Lugg. Instead of metaphosphoric acid we employed trichloroacetic acid (final concentration in extract 2·5%). As all our experiments were performed on solutions of pure compounds, the buffers employed were chosen to represent the concentrations of trichloroacetate and citrate which would be present in an extract adjusted to the requisite pH in the course of a determination by the HCHO technique. Also, in carrying out the indophenol titrations, we did not find it necessary to use the 'drop persistence' technique of Lugg. After the direct addition of the indicator the usual correction was made for the amount of dye required to produce the tint observed at the end-point.

Reagents

Formaldehyde—the solutions of formaldehyde were prepared from the Analar reagent by neutralizing the HCl present with NaOH, and diluting with water. The exact concentration was determined by a procedure based on Seyewitz & Gibello's method [1904], or by oxidation with H_2O_2 and titration of the acid formed.

l-*Ascorbic acid*, d-*glucoascorbic acid and reductic acid*—see Snow & Zilva [1941].

Reductone and dihydroxymaleic acid—see Snow & Zilva [1938].

d-*Araboascorbic acid*—kindly presented by Prof. E. L. Hirst. Brownish white crystalline powder. Purity 89% by indophenol titration.

6-*Desoxy*-l-*ascorbic acid*—kindly presented by Prof. T. Reichstein. Pure white crystals. Purity 100% by titration.

6-*Trityl*-l-*ascorbic acid*—prepared by the method of Vargha [1933]. The product prepared by us was similar in characteristics to that obtained by Vargha but its purity as determined by indophenol titration was only 70%.

5:6-*Isopropylene*-l-*ascorbic acid*—prepared by Vargha's method [1932]. Crude product recrystallized four times from acetone. Pure white; m.p. 208° (decomp.). Unlike Vargha, we found that this compound was not readily hydrolyzed in water. Vargha based his conclusion on the fact that in aqueous solution the molecular rotation of the isopropylene compound was identical with that of ascorbic acid. We also found these values to be similar but further work showed that this agreement was fortuitous. Thus when aqueous solutions were diluted with four volumes of ethanol the rotations of ascorbic acid and its acetone derivative were markedly different. It is seen (Table 1) that

Table 1. *Optical rotation of* 5:6-*isopropylene*-l-*ascorbic acid in aqueous and in alcoholic solutions*

(All measurements were made at 21° with light of wavelength 546 mμ.)

	Time before addition of alcohol to aqueous solution	*l*-Ascorbic acid		5:6-Isopropylene-*l*-ascorbic acid	
Solvent	(hr.)	Concentration (g./100 ml.)	[α]	Concentration (g./100 ml.)	[α]
Water	—	1·33	25°	1·63	27°
80% ethanol	0	0·27	52°	0·33	13°
	3½	—	—	0·33	14°
	7	—	—	0·33	16°

even after 7 hr. the change in rotation of the latter was so small that it was probably within the limits of the experimental error. The action of HCHO upon this compound could therefore be studied without any serious interference due to hydrolysis.

Buffer solutions.

	Vol. used to prepare buffers of pH	
	1·5	3·5
10% CCl_3.COOH solution	500 ml.	500 ml.
Citric acid (Analar)	24·7 g.	62·5 g.
10N NaOH (Analar)	22·6 ml.	56·8 ml.
Water to	1 l.	1 l.

The pH of each solution was checked electrometrically after addition of 27·5 ml. water and 5 ml. 6M HCHO solution/25 ml. buffer solution. If necessary, small additions of HCl or NaOH were made to the stock solutions to bring the pH after dilution to exactly 1·50 or 3·50.

Procedure. 25 ml. buffer solution and 30 ml. HCHO solution of the requisite concentration, contained in a 100 ml. flask, were placed in a thermostat and allowed to attain the temperature of the bath. As a precaution against atmospheric oxidation a current of O_2-free N_2 was passed through the reaction mixture during the experiment. An aqueous solution of ascorbic acid or other reducing compound (2·5 ml.) was added to make the initial concentration in every case 0·46 mM. Samples of the mixture were then withdrawn at intervals, acidified with HCl to arrest further reaction, and titrated with 2:6-dichlorophenolindophenol. The initial titration value of the reducing compound was determined separately. The results given are in every case the mean of at least two experiments and are expressed as percentages of the original concentration of the reactant.

Special modifications of this procedure were found necessary in the case of certain of the reducing substances employed. The estimation of reductone by direct titration is unsatisfactory because the rate of reduction of indophenol is too slow for the end-point to be easily observed. The reductone solutions were therefore added to an excess of acidified indophenol solution, allowed to stand for 1 min. and back-titrated with ascorbic acid solution stabilized with KCN, NaCl and acid. This method, although not entirely satisfactory in the presence of large concentrations of HCHO, was definitely preferable to the direct titration procedure.

Dihydroxymaleic acid in aqueous solution loses its reducing capacity on standing. In order to avoid this it was dissolved in absolute ethanol before addition to the buffer-HCHO mixture. It was also necessary to dissolve 6-trityl ascorbic acid in ethanol, owing to its low solubility in water.

In the case of alloxantin special precautions had to be taken to avoid oxidation by atmospheric O_2. Water and buffer solutions were boiled and cooled in a stream of N_2 and the reaction was conducted under strictly anaerobic conditions.

RESULTS

The kinetics of the reaction between formaldehyde and l-ascorbic acid

The reaction was studied over a range of temperatures and HCHO concentrations. It has been deduced from the data (Table 2) that the reaction is reversible and proceeds according to the equation $A+F \rightleftharpoons AF$ (A = ascorbic acid, F = HCHO, and AF = the complex formed from equimolecular proportions of the reactants). The forward reaction here is bimolecular and the opposing reaction unimolecular. Owing, however, to the high concentration of HCHO relative to that of the ascorbic acid present (23 molar equivalents at the lowest concentration used), the concentration of the former reactant may be considered constant, and the forward reaction therefore behaves as a reaction of the first order, with

$$\frac{dx}{dt} = k_1 (a-x)\,[F] - k_2 x .$$

From this may be deduced

$$k_1 [F] + k_2 = \frac{1}{t} \ln \frac{x_e}{x_e - x}$$

(a = initial concentration of ascorbic acid; x = amount combined in time t; x_e = amount of ascorbic acid combined at equilibrium; k_1 = velocity constant of forward bimolecular reaction; k_2 = velocity constant of opposing unimolecular reaction; and $[F]$ = concentration of HCHO). From the equilibrium equation the equilibrium constant (K) is obtained as follows:

$$K = \frac{[AF]}{[A]\,[F]} = \frac{k_1}{k_2}.$$

From the experimental results, values for K and $k_1 [F] + k_2$ could be derived, and hence the constants k_1 and k_2 could be calculated.

It will be seen from Table 2 that the figures for K and k_1 at a given temperature were constant within the limits of the experimental error, except for a slight deviation at the highest HCHO concentration at 30°; all values of K for the highest HCHO concentrations, however, must be regarded as somewhat unreliable owing to the smallness of the titration values from which they were derived. The general constancy of K and k_1 demonstrates that the reaction follows the postulated course over a considerable range of HCHO concentrations. An independent check was provided by plotting the values of $k_1 [F] + k_2$ against the HCHO concentration in the case of experiments carried out at 30·3°. The points lay along a straight line intersecting the axis at a point representing the value of k_2. The figure obtained in this way was 0·03, which was in good agreement with the value of 0·028 calculated from the equilibrium constant.

The variation of the velocity constants with temperature agreed with Lugg's results. At a HCHO concentration of 0·526 M, $k_{25°}/k_{15°} = 2·8$ ($k = k_1 [F] + k_2$). Lugg's corresponding figure was 3·0. In the presence of this large excess of HCHO, k_2 was almost negligible and values of

$$\log (k_1 [F] + k_2)$$

plotted against the reciprocal of the absolute temperature were found to lie on a straight line (Fig. 1). The same diagram also shows a similar rectilinear relationship when k_1, k_2 and K are plotted against $1/T$. The change in the equilibrium constant is of practical significance when the method is used under extreme conditions of temperature. The error introduced by temperature changes is much more serious at low HCHO concentrations, and it is probable that Lugg's failure to detect the variation was due to the high HCHO concentration present in his experiments.

Lugg's method is based on the fact that the velocity of combination of HCHO with ascorbic acid is much lower at pH 1·5 than at pH 3·5. In studying the course of the reaction at pH 1·5, only the early stages were considered by us, because of

Table 2. *The influence of temperature on the rate of combination, at* pH 3·5, *of ascorbic acid with varying concentrations of formaldehyde*

Initial ascorbic acid concentration, 0·46 mM. Equilibrium and velocity constants are expressed in mole, litre, minute units.

Temp. (°C.)	HCHO conc. (M)		Uncombined ascorbic acid after T min. (%)										Ascorbic acid combined at equilibrium (%)	Equilibrium constant K	Mean $k_1[F]+k_2$	k_1	$k_2 \times 10^3$
30·3	0·526	T	½	1	1½	2	3	4	6	8	10	20					
		%	54·0	29·3	16·0	8·7	4·1	2·8	2·3	2·0	2·0	1·8	98·0	(92)	1·30	(2·43)	
	0·132	T	2	4	6	8	12	16	20	30							
		%	51·7	28·1	17·1	12·0	8·6	7·4	7·1	7·1	—	—	92·9	100	0·37	2·60	
	0·0526	T	5	10	15	20	25	30	40	50	60	80					
		%	50·8	31·8	22·8	19·7	18·0	17·0	16·9	16·8	16·5	16·8	83·2	94	0·17	2·76	
	0·0263	T	10	20	30	40	50	60	80	100	120						
		%	54·5	37·7	32·1	29·6	28·6	28·4	28·2	28·6	27·8	—	71·8	97	0·099	2·72	
	0·0105	T	5	10	15	20	30	40	60	80	120	150					
		%	86·4	77·2	70·0	63·9	57·4	54·0	50·0	49·1	48·0	47·9	52·1	104	0·058	2·83	
												Mean		99	—	2·73	28
25	0·526	T	1	2	3	4	6	8	12	16	20						
		%	45·5	21·9	10·1	5·8	2·4	2·1	1·6	1·7	1·5	—	98·4	(115)	0·80	1·50	
	0·132	T	1	2	4	8	12	20	30	40	50						
		%	78·4	63·8	43·6	20·5	12·3	6·6	5·4	5·4	5·5	—	94·6	133	0·23	1·64	
	0·0263	T	4	8	16	30	50	80	120	150							
		%	83·2	71·5	54·3	36·6	26·9	23·4	22·8	22·4	—	—	77·6	132	0·057	1·67	
												Mean		133	—	1·60	12
20	0·526	T	1	2	3	4	6	8	12	16	20	30					
		%	62·8	38·4	24·0	15·7	6·9	3·5	2·0	1·5	1·5	1·3	98·5	(125)	0·49	0·92	
	0·132	T	2	4	8	12	16	24	40	60	80	100					
		%	77·3	58·6	38·1	24·5	16·2	9·0	5·1	4·5	4·3	4·3	95·7	171	0·13	0·96	
	0·0263	T	8	16	30	50	92	150	240	360							
		%	80·9	67·8	50·6	36·9	24·0	19·9	18·8	18·7	—	—	81·3	166	0·031	0·96	
												Mean		169	—	0·946	5·6
15	0·526	T	1	2	3	4	6	8	12	20	30	50					
		%	73·2	54·9	43·5	32·8	19·4	11·6	5·0	2·0	1·1	1·0	99·0	(189)	0·29	0·54	
	0·132	T	4	8	16	30	50	80	120	150							
		%	76·1	58·1	34·7	15·2	6·1	3·9	3·4	3·4	—	—	96·6	218	0·071	0·52	
	0·0263	T	30	60	90	120	180	270	390								
		%	70·5	49·9	37·0	28·6	20·3	16·3	15·2	—	—	—	85·3*	220	0·015	0·48	
												Mean		219	—	0·512	2·3
10	0·526	T	1	2	3	4	8	12	20	30	40	90					
		%	81·0	72·0	59·8	51·7	27·7	15·4	5·6	1·5	0·8	0·8	99·2	(247)	0·17	0·31	
	0·132	T	4	8	16	30	50	80	120	150	180						
		%	83·8	70·9	50·5	29·6	14·2	6·4	3·6	2·9	2·9	—	97·1	251	0·044	0·32	
	0·0263	T	30	60	150	240	330	420									
		%	76·7	60·6	32·0	20·1	15·7	13·7	—	—	—	—	87·8*	273	0·010	0·33	
												Mean		262	—	0·323	1·2

* Extrapolated.

(Figures in brackets not included in mean.)

the considerable time required to attain equilibrium. It followed that, at the high concentration of HCHO employed in all the experiments, the quantity of ascorbic acid combined at equilibrium could be assumed to be 100%, without the introduction of a serious error. The calculation was further simplified by neglecting k_2, which is very small under these conditions, and by accepting the expression $k_1[F]+k_2$ as the velocity constant (k) of a first order reaction. The validity of these simplifications was demonstrated in the case of the experiments at 30°, where measurements were continued almost to the point of equilibrium. It will be seen (Table 3) that the amount of ascorbic acid remaining was even smaller than under similar conditions at pH 3·5. When log k was plotted against $1/T$ (Fig. 1), the graph was a straight line with a slope only slightly different from that obtained for the same HCHO concentration at pH 3·5; $k_{25°}/k_{15°}=3{\cdot}1$.

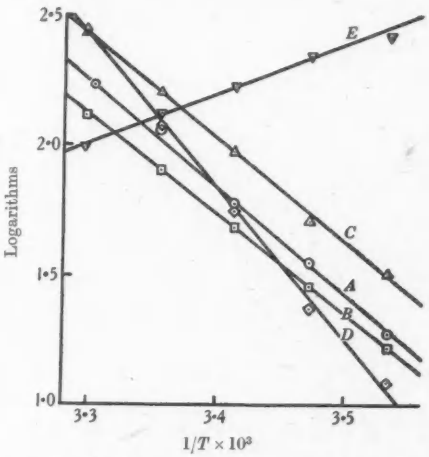

Fig. 1. Reaction between ascorbic acid and formaldehyde. Variation of equilibrium and velocity constants with temperature. (The data were derived from those given in Tables 2 and 3.) T = absolute temp. Curve A: pH, 1·5. $4+\log(k_1[F]+k_2)$. Curve B: pH, 3·5. $2+\log(k_1[F]+k_2)$. Curve C: pH, 3·5. $2+\log k_1$. Curve D: pH, 3·5. $4+\log k_2$. Curve E: pH, 3·5. $\log K$ (equilibrium constant).

It may be noted that we found a much greater difference between the velocities of reaction at pH 3·5 and 1·5 than that reported by Lugg. He found the velocity constants at pH 1·5 to be 0·03 times those at pH 3·5, whilst the ratio in our experiments was approximately 0·012 (varying slightly with temperature). From the figures given in his paper it would appear that the cause of the discrepancy lies in differences between the velocity constants obtained for the reaction at pH 1·5. The disagreement may possibly be explained by the difference in the composition of the solutions; in our experiments trichloroacetic acid was present, whilst Lugg employed metaphosphoric acid.

The kinetics of the reactions of formaldehyde with substances chemically related to l-*ascorbic acid*

The substances investigated were: *d*-araboascorbic acid, which differs from *l*-ascorbic acid only in the configuration of carbon 4; *d*-glucoascorbic acid, a seven carbon analogue; reductic acid, an alicyclic compound containing a keto-dienol group; reductone and dihydroxymaleic acid, both acyclic compounds with a dienol group, and alloxantin.

d-*Araboascorbic and* d-*glucoascorbic acids.* The velocity of reaction of these two compounds with HCHO (Tables 4 and 5) was similar to that of *l*-ascorbic acid, both at pH 1·5 and 3·5 (cf. Tables 2 and 3). At the higher pH value the reaction rapidly reached an equilibrium, but the

Table 3. *The influence of temperature on the rate of combination of ascorbic acid with formaldehyde at* pH 1·5

Formaldehyde concentration, 0·52 M. Initial ascorbic acid concentration, 0·46 mM.

Temp. °C.		Uncombined ascorbic acid after T min. (%) Velocity constant $k=(k_1[F]+k_2)10^3$										
29·7	T	4	8	12	16	20	60	120	180	270	360	
	%	92·9	87·2	82·1	76·4	71·0	35·1	13·3	5·5	2·1	1·5*	
	k	(18·4)	17·3	16·7	17·0	17·4	17·8	(19·4)	17·3	17·1	Mean	17·2
25	T	5	10	15	20	25	30	35				
	%	94·1	89·0	84·1	79·5	75·4	71·3	67·5				
	k	(12·0)	11·7	11·5	11·5	11·3	11·3	11·3			Mean	11·4
20	T	10	20	30	45	50	60	70	80			
	%	93·8	88·3	83·4	76·5	74·5	69·3	65·3	62·4			
	k	(6·45)	6·22	6·07	5·94	5·90	6·11	6·09	5·91		Mean	5·97
15	T	20	40	60	80	100	120					
	%	92·9	86·6	80·5	75·4	70·8	66·2					
	k	3·68	3·57	3·61	3·54	3·46	3·44				Mean	3·55
10	T	30	60	90	120	150	180					
	%	94·6	88·7	85·0	79·8	75·1	70·7					
	k	1·84	2·00	1·82	1·88	1·90	1·93				Mean	1·90

* % ascorbic acid at equilibrium = 1·1 (extrapolated). Figures in brackets not included in mean.

Table 4. *Reaction of various reducing substances with formaldehyde at* pH 3·5

Initial concentration of reducing compounds, 0·46 M. Temp. 25°

Reducing compound	HCHO conc. $=[F]$ (M)		Uncombined reducing compound after T min. (%) Velocity constant $k=k_1[F]+k_2$										Reducing compound combined at equilibrium $=x_e$ (%)	$x_e[F]^{-\frac{1}{2}}$
d-Glucoascorbic acid	0·526	T	½	1	2	3	4	8	12	20				
		%	58·9	38·5	15·6	6·0	2·9	0·7	0·5	0·5	—	—	99·5	
		k	(1·07)	0·96	0·94	0·97	0·94	Mean	0·95					
d-Araboascorbic acid	0·526	%	64·2	45·8	24·5	13·2	7·8	3·1	2·4	2·4	—	—	97·6	
		k	(0·92)	(0·81)	0·74	0·73	0·72	Mean	0·73					
Reductic acid		T	½	1	2	4	8	15	30	60	90	120		
	4·8	%	75·4	73·7	73·7	71·7	68·4	65·9	59·9	51·9	47·1	42·9	27	12
	2·4	%	83·6	83·6	84·1	81·1	79·8	78·6	75·1	70·1	67·9	65·9	18·5	12
	1·2	%	89·5	88·0	88·0	87·2	86·7	85·7	84·4	82·9	79·9	78·7	12·5	11
	0·60	%	94·4	93·2	93·2	93·4	92·7	90·9	91·4	90·2	88·9	87·9	8	10
Reductone		T	1	2	4	8	12	16	23					
	4·84	%	77	69	47	26	21	15	11					
		T	10	15	20	25	30	40	60	100	150			
	0·605	%	74	60	—	48	42	34	20	11	7			
	0·151	%	93	88	86	84	79	73	62	46	36			
Dihydroxymaleic acid		T	1	2	4	6	8	12	16	20	30	40		
	4·84	%	91·6	77·5	62·5	48·6	38·5	24·8	15·2	9·9	2·5	0		
		T	2	4	8	12	20	30	40	60	80	100		
	0·605	%	93·7	88·7	80·8	72·2	55·2	41·5	30·9	17·5	10·1	6·0		
		T	6	15	23	30	40	50	60	70	90	110		
	0·151	%	88·8	72·5	63·9	53·2	43·5	35·1	28·5	24·4	15·8	10·9		
		T	15	30	45	60	80	100						
	0	%	78·8	62·2	49·5	37·6	29·0	22·7						
5:6-Isopropylene-*l*-ascorbic acid		T	½	1	2	4	8	15	30	60	90	120		
	0·52	%	78·2	67·4	57·4	51·5	49·5	47·8	44·5	40·1	39·1	37·5	48·5	67
	0·26	%	86·5	80·3	71·1	65·6	62·3	60·4	57·8	54·0	51·0	48·8	36·5	72
	0·13	%	92·0	88·7	83·2	79·1	76·2	74·0	71·2	68·3	64·7	62·3	25	69
	0·065	%	94·0	92·4	89·3	86·2	84·6	82·5	78·7	76·0	74·4	71·6	19	75

(Figures in brackets not included in mean.)

Table 5. *Reaction of various reducing substances with formaldehyde at* pH 1·5

Initial concentration of reducing substances, 0·46 mM.

Reducing compound	HCHO conc. (M)	Temp. (°C.)	Uncombined reducing compound after T min. (%) Velocity constant $k=(k_1[F]+k_2)\,10^3$						
d-Glucoascorbic acid	0·52	25	T	5	10	15	20	25	30	
			%	94·2	89·0	84·7	79·2	74·9	69·6	
			k	12·0	11·7	11·1	11·6	11·5	12·1	Mean 11·7
Reductic acid	0·52	30	T	30	60	90	180	270	360	
			%	93·9	90·5	91·2	89·8	88·8	86·1	
Reductone	0·52	30	%	98	93	90	85	74	70	
Dihydroxymaleic acid	0·52	30	T	10	20	30	50	70	90	
			%	73·4	51·5	38·2	21·1	11·4	8·9	
	0		%	79·0	55·7	44·8	24·6	18·6	14·0	

amounts of the reducing substances combined differed somewhat from the corresponding values for *l*-ascorbic acid. The significance of this difference was, however, uncertain in view of the fact that neither of the two compounds was completely pure.

Reductic acid. The reaction of this compound at pH 3·5 was studied over a range of HCHO concentrations. As will be seen from Table 4, there was a rapid initial reaction which was followed by a very much slower one. The two stages can be clearly seen if the titration values are plotted against time. The rapid reaction is then represented by the steep initial part of the curve, the slope of which decreases sharply as this reaction ceases, and the slow reaction is indicated by the lower gradient of the curve as time proceeds. By extrapolating the flat portion of the curve back to the axis it was possible to determine the 'equilibrium' value of the initial reaction. The amount of reductic acid combined at this primary 'equilibrium' was far less than the equilibrium value for ascorbic acid at a corresponding HCHO concentration. The amount of reductic acid combined at 'equilibrium' varied with the HCHO concentration in a manner quite different from that found

with ascorbic acid. Owing to the uncertainty of the nature of the reaction involved it was not possible to calculate an equilibrium constant. An empirical relationship was, however, found to exist between the HCHO concentration and the percentage of reductic acid combined at equilibrium, i.e. $x_e \propto \sqrt{[F]}$ (symbols as in preceding section), as shown by the approximate constancy of the product $x_e[F]^{-\frac{1}{2}}$ (Table 4). The significance of this result is not apparent, and the question of the mode of combination of reductic acid with HCHO remains unsettled. It is, however, evident that at least two reactions are involved, one of which proceeds at a speed comparable with that of the ascorbic acid-HCHO reaction and quickly reaches an equilibrium.

It is probable from the figures given in Table 5 that the course of the reaction at pH 1·5 is quite similar to that at pH 3·5, though the rate of combination is very much slower.

Reductone. Lugg included this substance in the class of compounds which do not condense appreciably with HCHO either at pH 3·5 or 1·5. The results of our experiments at pH 3·5, with different HCHO concentrations, showed, however, that although the velocity of combination of reductone with HCHO was much lower than in the case of ascorbic acid, it was nevertheless significant. This was also found by Mapson [1943]. As the figures for this compound lacked the precision attained with other reducing compounds, the conclusions about the kinetics of this reaction were not so well defined. It was apparent, however (Table 4), that the loss of reductone was approximately exponential and continued until all, or at least a very large part, of this reactant was exhausted. It may be noted that the initial velocity of the reaction was approximately proportional to the concentration of HCHO present. The behaviour of reductone was thus completely different from that of reductic acid. Reductone also reacted with HCHO at pH 1·5, although under these conditions the rate of combination was very slow indeed (Table 5).

Dihydroxymaleic acid. Experiments on this compound were complicated by the instability of aqueous solutions of the substance, both at pH 3·5 and 1·5, even under completely anaerobic conditions, and a comparatively rapid loss of reducing power occurred without the addition of HCHO. The data (Table 4) show that at pH 3·5 the rate of loss of dihydroxymaleic acid in the presence of 0·15M HCHO was only slightly more rapid than in the absence of the latter. Higher concentrations of HCHO, however, caused the reaction to proceed more swiftly, and at a concentration of 4·84M no reducing capacity remained after 40 min. It thus seems possible that, apart from its spontaneous decomposition, the behaviour of dihydroxymaleic acid towards HCHO resembles that of reductone. From Table 5 it will be seen that at pH 1·5 the loss of the compound due to spontaneous decomposition occurred at a rate similar to that observed at pH 3·5, and was only slightly accelerated by HCHO at a concentration of 0·53M.

Alloxantin. Alloxantin combined very rapidly with HCHO both at pH 3·5 and 1·5; at a concentration of 0·52M HCHO, with pH 1·5, no reduction could be detected after 30 sec. at 25°. This compound thus undoubtedly belongs to Class 1 of Lugg's classification, which includes substances that readily condense with HCHO at pH 1·5.

Corrections for certain interfering substances

It is seen from the above that Lugg's method is incapable of distinguishing between *l*-ascorbic acid and its close analogues, and consequently no corrections could be made for their presence. On the other hand, *l*-ascorbic acid can be determined with some degree of accuracy in the presence of high concentrations of reductic acid, reductone or dihydroxymaleic acid if the necessary corrections are made.

In cases where the value obtained after treatment with HCHO at pH 3·5 is small in relation to that obtained by direct titration, no further correction is needed. If, on the other hand, this value be large, corrections for substances behaving like reductone and reductic acid are called for.

If the presence of reductone be suspected, it can be ascertained by oxidizing the extract with indophenol and subsequently reducing it with H_2S. By this treatment only a small fraction of the original reducing value of the reductone is regenerated, whilst the dehydroascorbic acid is completely reduced to ascorbic acid. However, this procedure may not diminish the error due to other substances which behave like reductone in the HCHO technique, and a general correction formula can be introduced in such cases.

The velocity of combination of reductone with HCHO is much lower than that of ascorbic acid at pH 3·5, but in the time required to bring the ascorbic acid reaction to within 0·5% of equilibrium approximately 17% of the reductone would combine. If, however, the experiment is repeated by doubling the reaction time, approximately twice as much reductone will combine, since the course of the reaction is almost linear in the early stages (Fig. 2);

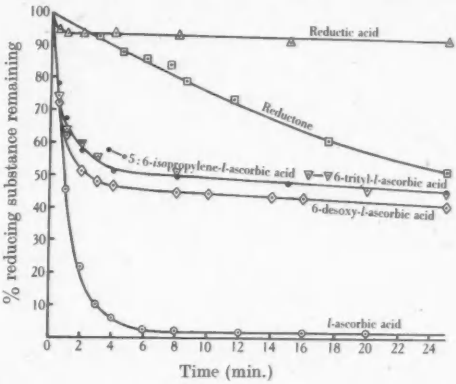

Fig. 2. Reaction of various reducing substances with formaldehyde at pH 3·5. Initial concentration of reducing substances, 0·46mM. HCHO concentration, 0·52M. Temp. 25°.

in both cases the ascorbic acid-HCHO reaction may be considered to have reached equilibrium. Thus the following method of calculation may be employed if reductone or substances behaving like it are present.

$$v_1 = v + w,$$

$$v_2 = v\left(1 - \frac{x_e}{100}\right) + w(1-p),$$

$$v_3 = v\left(1 - \frac{x_e}{100}\right) + w(1-2p),$$

whence $$v = (v_1 - 2v_2 + v_3)\frac{100}{x_e}$$

(v_1 = corrected titration after reaction with HCHO at pH 1·5; v_2 = titration after reaction at pH 3·5 for standard

time (t); v_2=titration after reaction at pH 3·5 for time $2t$; v=titration equivalent of actual ascorbic acid present; w=titration equivalent of reductone present; x_e=percentage of ascorbic acid combined at equilibrium, and p=fraction of total reductone combined in time t).

This procedure is similar to that adopted by Mapson [1943]. Mapson's modification has the advantage that it does not require special care in the adjustment of the pH and the concentration of HCHO, and is independent of any changes in the reaction velocity at pH 3·5. On the other hand, it requires more material and time for the estimation, and above all, as in the case of Lugg's original method, no correction is made for the error that might arise from the presence of reductic acid.

As the initial velocity of combination of reductic acid with HCHO is comparable with that of ascorbic acid, the procedure recommended for use with reductone and like substances is of no value in this case. However, use may be made of the fact that the amount of reductic acid combined at 'primary equilibrium' is proportional to $\sqrt{[\text{HCHO}]}$. After the usual HCHO technique at pH 3·5 is carried out, the procedure is repeated with one-fourth of the concentration of HCHO, the reaction time being suitably increased so as to allow the ascorbic acid-HCHO system to reach equilibrium. In this second titration the percentage of reductic acid combined will be approximately one-half of that in the first titration.

$$v_1 = v + y,$$

$$v_2 = v\left(1 - \frac{x_e}{100}\right) + y\,(1 - q),$$

$$v_4 = v\left(1 - \frac{x_e'}{100}\right) + y\,(1 - \tfrac{1}{2}q),$$

whence

$$v = \frac{100\,(v_1 - 2v_4 + v_2)}{2x_e' - x_e}$$

(v_2=titration after reaction at pH 3·5 with standard HCHO concentration (c); v_4=titration after reaction at pH 3·5 with HCHO concentration 0·25c; y=titration equivalent of reductic acid present; q=fraction of reductic acid combined at HCHO concentration c; x_e=percentage of ascorbic acid combined at equilibrium with HCHO concentration c; x_e'=percentage of ascorbic acid combined at equilibrium with HCHO concentration 0·25c; v_1 and v have the same significance as before).

Values for the reaction times and the percentage of ascorbic acid combined at equilibrium for the two HCHO concentrations are given in Table 6, in order that this correction may be applied.

The presence of a substance like dihydroxymaleic acid, which decomposes spontaneously both at pH 3·5 and 1·5, would undoubtedly lead to erroneous results in applying Lugg's method, if the extracts were not previously treated with H_2S. If, however, the solutions are allowed to stand in the presence of H_2S, this interfering substance will decompose before the estimation is commenced, and error due to its presence will thus be avoided.

Table 6. *Time required for the combination of ascorbic acid with formaldehyde at* pH 1·5 *and* 3·5

$[F]$=concentration of HCHO in reaction mixture.
x_e =percentage of ascorbic acid combined at equilibrium.
At pH 1·5, times given are those required to produce the percentage loss indicated.
At pH 3·5, times given are those required to reach 99·5% equilibrium value.

	pH 1·5. Time with $[F]$=0·52M		pH 3·5			
			$[F]$=0·52M		$[F]$=0·13M	
Temp. (° C.)	1% loss (sec.)	5% loss (min.)	Time (min.)	x_e	Time (min.)	x_e
30	35	3	4	98·2	15	93·0
25	60	5	7	98·5	24	94·4
20	100	8·5	11	98·9	40	95·6
15	170	15	19	99·1	68	96·5
10	310	26	32	99·3	119	97·3

(The data are derived from those in Tables 2 and 3.)

The structure of the formaldehyde-ascorbic acid complex

It has already been seen from the kinetics of the reaction, that HCHO and ascorbic acid combine in equimolar proportions. It is therefore probable that the HCHO molecule has two points of attachment in the ascorbic acid molecule. An attempt to isolate the complex, by evaporating concentrated aqueous solutions or by fusing ascorbic acid with polymers of HCHO, did not meet with much success. Rigorous drying gave almost colourless glassy solids which deliquesced immediately on exposure to air and which contained a small amount of unchanged ascorbic acid. When their aqueous solutions were made alkaline and immediately acidified, a large part of the indophenol titration value of the combined ascorbic acid was regenerated. This will be seen later to be characteristic of the complex. Thus these impure solid products contained a considerable proportion of material which behaved in a manner similar to the ascorbic acid-HCHO complex produced in solution. Purification of the solids was rendered difficult by their extremely deliquescent nature, and, in view of the probable complexity of the ascorbic acid-HCHO reaction, further attempts to isolate the complex were felt to be unprofitable. The problem of structure had therefore to be attacked by indirect methods.

The fact that after combination with HCHO the ascorbic acid loses its reducing properties suggests that either or both of the OH groups attached to C_2 and C_3 are involved. Our first object was therefore to inquire into the nature of this linkage. If the condensation were to involve C_3, it should lead to a decrease in the acidic properties of ascorbic acid, since the H atom in this position is probably the one which is mainly ionized [Reichstein, Grüssner & Oppenauer, 1934; Haworth & Hirst, 1934; Haworth, Hirst & Smith, 1934]. In such a case the pH of an unbuffered mixture of HCHO and ascorbic acid should increase as the reaction proceeds. To test the point the following experiment

was carried out. 20 ml. 6M neutral HCHO solution were added to a solution of 0·5 g. ascorbic acid in 180 ml. water at room temperature. The pH of the solution was determined initially and after various intervals by means of a glass electrode system. Simultaneously samples of the mixture were titrated with indophenol. In correlating the pH value and the ascorbic acid concentration of the reacting solution, use was made of a curve previously constructed by measuring the pH of aqueous solutions of known strengths of ascorbic acid. It will be seen from Fig. 3 that although the observed pH values were

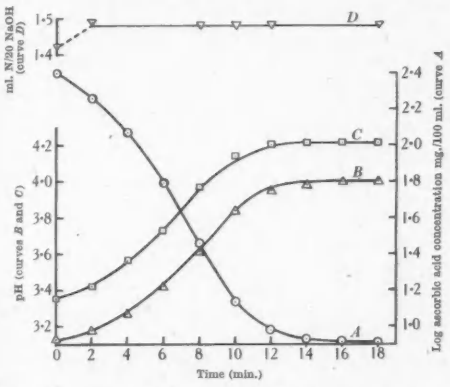

Fig. 3. Changes in pH during reaction between ascorbic acid and formaldehyde. Reaction mixture—0·5 g. ascorbic acid, 180 ml. water, 20 ml. 6M neutral HCHO solution. Temp. 20°. Curve *A*: log ascorbic acid concentration (mg./100 ml.), from indophenol titrations. Curve *B*: pH calculated from ascorbic acid concentration. Curve *C*: pH measured electrometrically. Curve *D*: titration of 5 ml. portions with N/20 NaOH.

somewhat higher than those calculated from the titration values, the changes occurring as the reaction proceeded followed a very similar course in each case. The agreement was quite satisfactory in view of the ease with which the pH of an unbuffered solution may be affected by extraneous substances. The higher pH values were in all probability due to the presence of HCHO. In our opinion this experiment produces sufficient evidence that C_3 is one point of linkage.

In order to determine the position of the second point of attachment, the behaviour of certain derivatives of ascorbic acid containing substituents in positions 5 and 6 was investigated. The compounds employed were 6-trityl-, 6-desoxy- and 5:6-*iso*propylene-*l*-ascorbic acid, and the reaction of each of these substances with HCHO at pH 3·5 was followed up by titration as before. It will be seen (Fig. 2) that the behaviour of the three compounds was remarkably similar, and was in sharp contrast to that of ascorbic acid at the same concentration of HCHO. The course of the reaction was not unlike that observed with reductic acid, though the amount of reactant combined during the initial rapid reaction was much greater at a given HCHO concentration in the case of the 6-substituted compounds. The reaction with 5:6-*iso*propylene-ascorbic acid was investigated over a range of concentrations of HCHO. As in the case of reductic acid the expression $x_e\,[F]^{-\frac{1}{2}}$ was found to be approximately constant (Table 4). Since the behaviour of the three compounds was very similar in spite of the different nature of the substituents, it may be inferred that the contrast between the course of their reactions and that with ascorbic acid was probably not due to any change in the reactivity of the dienol H atoms, but to the blocking of position 6. Also since the 5:6 substituted *iso*propylene derivative behaved exactly like the 6-substituted compounds, linkage at position 5 may be excluded. It thus seems probable that the formation of the HCHO complex involves the 3 and the 6 positions of the ascorbic acid molecule. The possibility that the presence of HCHO might stabilize an abnormal tautomeric form of ascorbic acid having an oxygen bridge attached at C_6 (cf. the structure of *iso*dimethyl ascorbic acid [Micheel & Schulte, 1935; Micheel & Bischoff, 1936; Haworth, Hirst, Smith & Wilson, 1937]) appears to us very unlikely.

In all these experiments it must be emphasized that we have been concerned only with the HCHO condensations which affect the reducing power of ascorbic acid. A few experiments were carried out in an attempt to follow the course of the reaction between ascorbic acid and HCHO by observation of the changes in the optical rotation. The results indicated that the whole reaction system was more complicated than that indicated by titration methods. It is quite probable that other reactions may occur, leading to products which are still capable of reducing indophenol. As the presence of such compounds would have no bearing on the method, the matter was not investigated further.

In connexion with the properties of the ascorbic acid-HCHO complex, we should like to draw attention to a point of interest. In the investigation of the change in pH of the unbuffered ascorbic acid-HCHO solution in the above experiment, samples of the solution were titrated with NaOH (phenolphthalein indicator). Apart from a slight initial rise the titration values remained constant (Fig. 3) throughout the experiment. It would appear from this that the ascorbic acid-HCHO complex is very rapidly decomposed by alkali. This idea was confirmed by the fact that when the alkali-titrated solution was acidified and titrated with indophenol the quantitative regeneration of ascorbic acid was established.

The influence of various substances on the velocity of combination of ascorbic acid and formaldehyde

Since Lugg's method depends upon the rate of combination of ascorbic acid with HCHO at pH 3·5 and 1·5, it is evident that the accuracy of the method would be diminished by the presence of substances capable of influencing the velocity of the reaction. At pH 3·5 the reaction is allowed to reach equilibrium. The times suggested by Lugg are probably greater than is necessary to attain this condition and consequently the possibility of error is reduced. Thus a moderate decrease in the reaction velocity would not be serious, whilst an increase would have no effect upon the result. At pH 1·5, on the other hand, the reaction is allowed to proceed until a definite fraction of the ascorbic acid present (5% in Lugg's precedure) has combined with HCHO, and consequently any variation in the velocity of reaction at this pH would cause some error in the results. This error might be considerable if the reaction were accelerated. We found that when ascorbic acid was added to extracts of certain canned products the rate of reaction with HCHO at pH 1·5 was in fact considerably higher than in pure buffer solutions. Evidently this acceleration was produced by the presence of interfering substances in the extract and we therefore considered it advisable to investigate the influence of some compounds likely to be present in such extracts, as well as that of compounds capable of reacting with HCHO. For this purpose the substances were dissolved in buffer solution containing ascorbic acid and the HCHO solution added when the temperature was stabilized. Care was taken to ensure that the pH of the final mixture was 1·50. Results of these preliminary experiments are given in Fig. 4. It will be seen that, at a concentration of 10mM., any changes in the rate of reaction caused by creatine, *l*-tyrosine, asparagine or leucine were too small to be regarded as significant. On the other hand the presence of glycine (Analar reagent, twice, recrystallized from water), at the same concentration, more than doubled the initial velocity; at higher concentrations the increase was even greater. It must, however, be noted that, whilst the effect with glycine was most marked in the initial stages of the reaction, the increase of velocity observed with the extracts of canned material continued throughout the reaction. The problem requires further investigation, but these results definitely suggest that caution must be exercised in interpreting the results obtained at pH 1·5. If the difference between the direct titration and the values obtained after treatment with HCHO at pH 1·5 is small, no serious error can arise. If, however, the difference is considerable, an inquiry must be instituted in order to ascertain whether this difference is due to the presence of a substance in Class 1 of Lugg's classification or to an increase in the velocity of combination of ascorbic acid with HCHO, brought about by some substance in the extract. It may be noted that many of the substances which are included in Class 1 react almost instantaneously with HCHO at pH 1·5 and therefore the reaction times suggested by Lugg are probably longer than is necessary. We usually allow time for 1% of ascorbic acid to combine instead of 5%. In this way any errors due to changes in the reaction velocity are greatly reduced.

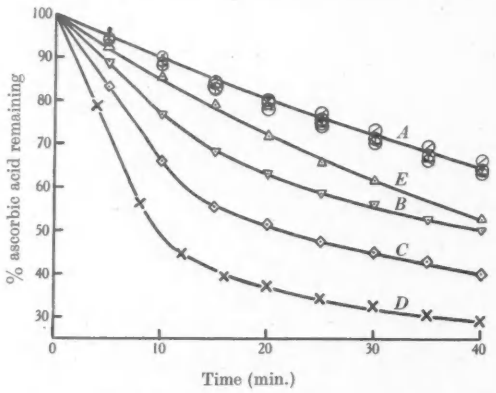

Fig. 4. The influence of various substances on the rate of combination of ascorbic acid with formaldehyde at pH 1·5. Initial concentration of ascorbic acid, 0·46mM. Temp. 25°. HCHO added at zero time. Curve *A*: • No addition; ⓘ 10mM. creatine added; ⊘ 10mM. tyrosine added; ⊘ 10mM. asparagine added; ⊖ 10mM. leucine added. Curve *B*: ▽ 10mM. glycine added. Curve *C*: ◇ 20mM. glycine added. Curve *D*: × 40mM. glycine added. Curve *E*: △ Loss of added ascorbic acid from extract of canned meat and vegetables adjusted to pH 1·5.

The differential behaviour of the oxidation products of ascorbic acid, reductic acid and reductone

It was mentioned in the introduction that at the time of the appearance of Lugg's paper we were engaged in elaborating methods for the determination of ascorbic acid in the presence of reductic acid and reductone. Our experiments were based on our observation that the primary oxidation products of the three compounds possessed certain characteristics which were distinctive enough to enable a separation to be effected. Dehydroreductone could be readily distinguished from dehydroascorbic acid by the fact that only a slight indophenol titration was obtained from the former on treatment with

H_2S at pH 3·5–4·5. Ascorbic acid could therefore be determined, in a mixture of these two compounds, by oxidation and reduction. Reductic acid could be distinguished from ascorbic acid by the greater stability of solutions of dehydroreductic acid at a low pH as compared with those of dehydroascorbic acid, and also because in the oxidized state the former reacted with 2:4-dinitrophenylhydrazine more rapidly than the latter. The following is a short description of the procedure adopted.

(*a*) Sufficient HCl was added to the oxidized solutions to bring the concentration to N/10. The acidified solution was then heated for 1 hr. at 100°, cooled, brought to pH 4·0 by addition of sodium acetate solution, reduced with H_2S and titrated with indophenol, in the usual way, after removal of the excess of H_2S. Under these conditions approximately 89% of the dehydroascorbic acid and only 3% of the dehydroreductic acid were destroyed. In practice the accuracy of the method was limited by the tendency for end-points to be indistinct after the acid treatment. Ascorbic acid mixed with 0·1–10M equivalents of reductic acid could be determined with an error not greater than 10%. For use with tissue extracts it would be necessary to introduce a correction for reductic acid produced, from pectins or pentoses, during the acid treatment, which could be effected by titrating a portion of the solution before reduction with H_2S. It is possible that a more satisfactory procedure could be devised at a lower temperature (40–50°) and higher acid concentration [cf. Penney & Zilva, 1943].

(*b*) The oxidized solution which contained approximately 0·5mM. dehydroascorbic acid or dehydroreductic acid was buffered at pH 1·5. 10 ml. were treated with 2 ml. 0·2% 2:4-dinitrophenylhydrazine in N HCl, and 1 ml. 2M sodium acetate (to neutralize the acid in the former reagent). The mixture was incubated for 80 min. at 38°, 2 ml. 0·28% aqueous furfural solution added (to remove excess of the hydrazine reagent), and the incubation continued for a further 10 min. Finally 3 ml. acetate buffer were added to bring the pH to 4·0 and the precipitate removed by filtration. The filtrate was reduced with H_2S and subsequently titrated in the usual way. In the hydrazine treatment approximately 6·5% of the dehydroascorbic acid and 96% of the dehydroreductic acid were lost. Ascorbic acid mixed with 0·1–10M equivalents of reductic acid could be determined with an error of less than 4%. In attempting to apply this procedure to extracts of biological materials we encountered difficulty owing to the formation of colloidal solutions of the dinitrophenylhydrazones.

DISCUSSION

There is little doubt that Lugg's procedure greatly improves the specificity of the indophenol titration for *l*-ascorbic acid. The specificity is, however, not absolute and critical judgement has to be employed in interpreting the results. Apart from the fact that *l*-ascorbic acid cannot be distinguished from its close analogues by this method, every determination must be considered in relation to the circumstances of any given case, in order to correct for the possible presence of interfering substances. These compounds may either belong to a category of compounds capable of reducing indophenol, or may exert an influence on the rate of combination of *l*-ascorbic acid with HCHO.

In applying Lugg's method the following points have therefore to be borne in mind: Lugg assumes that, when equilibrium is reached, 2% of the ascorbic acid remains uncombined. Our results show that the amount of ascorbic acid at equilibrium depends on temperature (Table 6). This point is of practical importance if the determination is carried out at extreme temperatures. Furthermore, the reaction is very sensitive to slight changes in pH. Although fair accuracy can be obtained under favourable conditions with indicators (methyl orange, thymol blue), the use of a glass electrode system for the control of the pH is desirable. It must also be pointed out that a change in pH may occur on the addition of HCHO. The concentration of HCHO is also important, and the strength of the reagent should be determined before use. It is important, as suggested by Lugg, to pass N_2 through the solutions during the determinations.

Under the conditions employed by us, the times required for the reactions at pH 1·5 and at 3·5 differed somewhat from those given by Lugg. In Table 6 are given the times required for 1 or 5% of ascorbic acid to combine with HCHO at pH 1·5. At pH 3·5, the times required for the reaction to proceed to 99·5% of the equilibrium value are given. In practice these times are multiplied by a factor of 1·5 in order to avoid an error due to any slight decrease in the velocity of the reaction that may occur.

Lugg's procedure depends for its accuracy upon the constancy of the velocity of the reaction between ascorbic acid and HCHO. A diminution in the velocity would not be serious unless it were sufficient to prevent the establishment of an equilibrium at pH 3·5 in the time chosen. A considerable acceleration of the reaction, however, would affect the results obtained at pH 1·5. This is a point of practical significance, since it has been found that certain extracts can increase the reaction velocity, and an increase can also be brought about by the addition of glycine. Such errors may be reduced, although not eliminated, by reducing the reaction times employed.

Lugg's method greatly reduces the error in the titration method due to the presence of reductone

or reductic acid, when present in quantities which are small in relation to that of the ascorbic acid present. When larger quantities of these compounds are present, additional corrections must be introduced.

SUMMARY

1. The kinetics of the reaction between HCHO and *l*-ascorbic acid or chemically related substances have been studied.

2. Indirect evidence is produced for the possible structure of the HCHO-*l*-ascorbic acid complex.

3. Lugg's method for determination of *l*-ascorbic acid has been examined from the point of view of the above experiments, and necessary precautions are stressed.

4. Suggestions for other methods for the determination of *l*-ascorbic acid in the presence of reductic acid and reductone are advanced.

One of us (G. A. S.) is indebted to the Medical Research Council for a whole time grant. We should like to express our gratitude to Prof. E. L. Hirst for specimens of *d*-araboascorbic and *d*-glucoascorbic acids, and to Prof. T. Reichstein for a specimen of 6-desoxy-*l*-ascorbic acid. Thanks are also due to Roche Products Ltd. for a gift of *l*-ascorbic acid.

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Studies in Immunochemistry

7. THE ISOLATION FROM HOG GASTRIC MUCIN OF THE POLYSACCHARIDE-AMINO ACID COMPLEX POSSESSING BLOOD GROUP A SPECIFICITY

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Investigations on the nature of the blood group antigens present in human tissues and secretions are greatly restricted owing to the small amounts of active factors present and to the difficulty of obtaining the tissues and secretions in large quantities. An observation of Schiff [1930], however, revealed that commercial peptone contained a considerable quantity of a blood group A factor, and subsequently Schiff & Weiler [1931], Brahn, Schiff & Weinmann [1932] and Schiff [1933] showed that commercial pepsin likewise could serve as a readily available source of this blood group substance. A rich supply of the A-factor was discovered later in hog gastric mucin by Landsteiner & Chase [1936] and by Meyer, Smyth & Palmer [1937]. As a result of their work on the glyco-proteins occurring in gastric mucosa, Meyer *et al.* [1937] isolated from this tissue a polysaccharide that possessed intense A activity and showed that it contained N-acetylglucosamine and galactose in equimolecular quantities. On the basis of the glucosamine content the product was considered to be only 75% pure, and it was observed that the material gave a positive Ehrlich diazo-reaction. Landsteiner & Harte [1940] confirmed these observations and extended the investigation to include a closer examination of the non-hexosamine nitrogenous constituent. They concluded that the A-substance consisted largely of carbohydrate but contained also a component rich in amino-acids.

The A-substance in hog gastric mucin is closely related serologically to the human blood group A factor. In view of the ease with which considerable amounts of hog gastric mucin could be obtained, it seemed a particularly suitable material for experimental study, the results of which could be employed subsequently as a basis for the elaboration of a method for the isolation of the blood group substances from human erythrocytes, tissues and tissue fluids. Cellular and tissue antigens, however,

are usually found to be labile complexes and it is becoming increasingly evident that if their isolation is to be achieved with the minimum of change in the physical, chemical and biological properties, it is essential to avoid conditions that involve extreme pH values, and to work with the material, as far as practicable, at low temperatures. The employment of these restricted conditions for the manipulation of molecular species of biological origin enables certain irreversible changes to be avoided or reduced to a minimum. With the above considerations in mind two methods have been elaborated for the isolation of the specific A-substance from commercial gastric mucin.

EXPERIMENTAL

Isolation of the specific A-substance from gastric mucin

Method 1. Commercial gastric mucin, obtained from the Wilson Laboratories, Chicago, was suspended in saline or in water saturated with CO_2. The material, at a concentration of about 2%, was vigorously shaken and kept saturated with CO_2, until a thick viscous solution was obtained. Usually one or two days were required for the small hard particles of the crude dry gastric mucin to soften, swell and finally pass into solution. The thick, viscous suspension, at a concentration of about 1%, was centrifuged in the closed bowl of a Sharples centrifuge for 1 hr., and the slightly opalescent supernatant solution was then carefully withdrawn from the bowl by gentle suction. About a third of the original crude gastric mucin remained undissolved. The insoluble material was washed by suspending in saline or in water saturated with CO_2, being washed four times in this manner, and finally dried *in vacuo* (fraction 1, Table 1). The material showed no appreciable A activity as measured by either the haemolysis inhibition or isoagglutination inhibition test. The main aqueous extract, together with the washings after they had been concentrated to small volume, was warmed to about 35°, and 30% by weight of anhydrous Na_2SO_4 was added, in small portions at a time, to the vigorously stirred solution. The A-substance separated sharply between 27 and 30% of Na_2SO_4, as a sticky gelatinous mass, and was collected on the centrifuge. The material was suspended in water and after solution was reprecipitated with Na_2SO_4. In some preparations the A-substance was treated with Na_2SO_4 a third time. The final precipitate was dissolved in water, dialyzed at 0° until free from SO_4^{--}, spun to remove any insoluble residue, and dried *in vacuo* from the frozen state (fraction 2, Table 1).

Table 1. *The fractionation of an aqueous solution of crude gastric mucin by sodium sulphate precipitation*

Nature of fraction	Amount of fraction* (g.)	$[\alpha]_{5461}$ (c, 0·5%)	Relative viscosity η	Total N (Kjeldahl) (%)	Ash (%)	Approximate minimum quantity (μg.) inhibiting completely: (a) Isoagglutination	(b) A-haemolysis
(1) Insoluble in water, soluble in alkali	8·6	−45°	1·14†	11·2	3·8	100–200	—
(2) Precipitated from aqueous solution between 27–30% Na_2SO_4	16·0	+ 8°	2·67	6·1	1·5	0·1	0·01
(3) Soluble in 30% Na_2SO_4	3·0	−22°	1·22	3·8	3·9	25	2

* From 40 g. of a specimen of crude gastric mucin, of which 23% was diffusible through a cellophane membrane.
† In 0·05N NaOH.

Measured by its power to inhibit isoagglutination, fraction 2 is the most active A-substance yet obtained by us. Other constituents (fraction 3, Table 1) of the gastric mucin which were recovered from the Na_2SO_4 supernatant fluids showed much reduced capacity to inhibit the isoagglutination or haemolysis of A-erythrocytes by anti-A serum. The results of fractionating 14·5 g. of fraction 3 from aqueous solution by ethanol are set out in Table 2. The fractions show only slight A activity, which amounts at the most to only a few per cent of the activity of the purified A-substance. This material closely resembles the acid polysaccharide described by Meyer *et al.* [1937]. Further fractionation gave a main fraction with the following properties, $[\alpha]_{5461}$, −34°; N, 3·9%; limit of inhibition of isoagglutination, 1:20,000.

Table 2. *The fractionation by ethanol precipitation of the* 30% Na_2SO_4 *soluble material* (14·5 *g.*) *obtained from crude gastric mucin*

Fraction no.	Limits of ethanol concentration for precipitation (% (v/v))	Weight of fraction (g.)	$[\alpha]_{5461}$ (c, 0·5%)	Relative viscosity η	Total N (%)	Approximate dilution showing complete inhibition of: Isoagglutination	A-haemolysis
1	—	1·2	+36°	1·07	11·1	$1:10^4$	$1:10^5$
2	0–20	1·7	+ 4°	1·08	9·4	$1:10^4$	$1:2\times10^5$
3	20–33	2·7	−14°	1·16	3·8	$1:4\times10^4$	$1:6\times10^5$
4	33–50	3·2	−15°	1·30	4·5	$1:4\times10^4$	$1:10^6$
5	50–66	4·5	−32°	1·19	4·0	$1:8\times10^4$	$1:2\times10^6$

Method 2. Crude gastric mucin was ground in a mortar with 90% phenol. The solvent was added gradually, the material being worked up first to a paste, then to a thick suspension. A total volume of about 2 l. of phenol was used for each 100 g. of gastric mucin, and the final suspension was allowed to stand, with frequent shaking at room temperature, for 24–48 hr. The crude mucin was partly soluble, the insoluble portion being separated from the viscous solution by spinning in the Sharples centrifuge. The insoluble material from each 100 g. of crude mucin was resuspended in about 500 ml. of 90% phenol, allowed to stand for 12 hr., and again recovered from the phenol solution. After a further treatment with phenol this residue was washed with ethanol to remove phenol and dried *in vacuo* (fraction 1, Table 3). The main phenol supernatant fluid, together with the phenol washings from the phenol-insoluble residue, was treated with ethanol to precipitate the A-substance. The ethanol was added as a 1:1 phenol-ethanol mixture in order to avoid local precipitation of the A-substance, which was thrown out of solution as a gelatinous mass when the ethanol concentration reached 10% (v/v). The precipitate was collected, redissolved in half the original volume of 90% phenol, centrifuged to remove any undissolved material and reprecipitated with ethanol. Solution in phenol and reprecipitation with ethanol were both repeated a third time. The final clear, almost colourless jelly was immediately dialyzed at 0°, or was triturated with ethanol to remove excess phenol, and the resulting friable mass then suspended in water and dialyzed at 0° for several days to eliminate the last traces of phenol and ethanol. The clear viscous solution was then frozen at −20° and dried *in vacuo* from the frozen state (fraction 2, Table 3).

The properties of the other fractions obtained by the addition of ethanol to the pooled 10% ethanol solutions are also given in Table 3. The material precipitated from the 10% ethanol-phenol supernatant fluid on raising the ethanol concentration to 25% (v/v) contains an appreciable amount of A-substance which can, however, be readily recovered by repeating the fractionation process. It will be seen that the specific activity, as measured by the capacity of the fractions to inhibit isoagglutination or the haemolysis of sheep cells by anti-A red cell serum, is much less than for the main A-substance fraction (fraction 2), which separated from the 90% phenol solution between 0 and 10% ethanol.

After the preliminary experiments had been completed, two preparations of the A-substance were made by method 1 and three large preparations by method 2. The yields of the final product were in each instance about 33% of the crude gastric mucin and in all the experiments described later in this paper it has not been possible to distinguish between the purified materials isolated by either of the two methods.

A preparation of mucin purchased in this country and purified by the two methods described above yielded purified A-substances that were very similar in properties to the A-substance described in this paper. The yield from the crude mucin was about 50%, the specific rotation of the purified material was, however, slightly less dextrorotatory, $+2° \pm 2°$ and the N was 4·9% or 5·1% according to the method of preparation used. The specimens gave rise to inhibition of isoagglutination at a dilution of $1:1·28 \times 10^6$. The relative viscosity (η, 2·45) was rather less than the value given for the A-substance derived from Wilson's gastric mucin.

Table 3. *The fractionation of crude gastric mucin from* 90% *phenol by ethanol*

Details of fraction	Amount of fraction* (g.)	$[\alpha]_{5461}$	Relative viscosity in 0·9% NaCl†	‡ Total N (%)	Ash (%)	Approximate minimum quantity (μg.) inhibiting completely: (a) Isoagglutination	(b) A-haemolysin
(1) Insoluble in 90% phenol	36·9	+27°§	1·25	6·9‖	14·7	10–100	10–100
(2) Precipitated from 90% phenol by ethanol between 0–10% concentration	38·2	+10°	2·86	5·5	0·9	0·1	0·01
(3) Precipitated from 90% phenol by ethanol between 10–25% ethanol	20·7	− 4°	1·24	8·0	2·0	1·0	0·1
(4) Precipitated from 90% phenol by ethanol between 25–50% ethanol	13·5	−67°	1·14	12·2	2·1	10	20
(5) Soluble in 50% ethanol phenol	14·7	−82°	1·05	10·4	5·2	No appreciable activity	

* From 145 g. of crude gastric mucin, of which, on dialysis, about 20% passed through a cellophane membrane.
† Relative viscosity of 0·9% NaCl taken as 1·00.
‡ Determined by Kjeldahl's method.
§ In 0·1 N NaOH.
‖ Uncorrected for ash.

RESULTS

Physical and chemical properties of the A-substance

The material obtained by drying a neutral aqueous solution of the A-substance from the frozen state is of a white, silky, almost crystalline appearance. It dissolves readily in water or saline, and a 1% solution shows considerable viscosity and is faintly opalescent. A 2–3% solution behaves as a viscous solution with pronounced elastic properties, and at a concentration of 4–5% the viscous solution fails to flow out of an inverted test-tube.

Several preparations were examined after solution in buffer acetate (pH 4·0) or phosphate (pH 8·0) buffer (μ, 0·1), in the Tiselius electrophoresis apparatus, by our colleague Dr R. A. Kekwick, who reported (see Addendum) that specimens obtained by either method were essentially homogeneous. Electrometric titration of an aqueous solution of the A-substance failed to reveal any group ionizing between pH 2·0 and 10·5.

The analytical figures for these preparations are recorded in Table 4. The amount of organically bound P was between 0·05 and 0·1 % in all preparations. Sulphur was absent. The acetyl estimations were carried out by the method of Elek & Harte [1936], but, although accurate and reproducible results were obtained with N-acetylglucosamine, the results with A-substance differed by as much as 15 %. The source of error was not detected, although the possibility of acyl residues other than acetyl was considered. Acetic acid was isolated as Ag salt (found, Ag 64·6; calc. 64·7 %) from an acid distillate of A-substance. The yield was of the same order (75 %) as was obtained in similar estimations on acetic acid.

Table 4. *The analytical data for preparations of the A-substance isolated from gastric mucin*

Method of preparation and no.	Weight of preparation (g.)	$[\alpha]_{5461}$ (0·5 % in H_2O)	Relative viscosity (0·5 % in saline)	Content (%) of C	H	N*	Ash	Acetyl
Phenol extraction								
GM. 201	30	+11°	2·80	—	—	5·7	1·4	9·6
GM. 210	33	+14°	2·86	45·2	6·6	5·9	0·9	10·7
GM. 240	38	+10°	2·83	45·1	6·7	6·0	0·9	10·3
Aqueous extraction								
GM. 280	9	+ 8°	2·78	44·8	6·4	6·4	0·8	9·2
GM. 321	20	+12°	2·35	45·9	6·4	6·2	0·6	11·5

* By Dumas's method.

Viscosity. The relative viscosity values of 0·5 % solutions of the preparations in 0·9 % NaCl were determined at 37° by means of a modified Ostwald viscosimeter. For the three large preparations of A-substance extracted by means of phenol, the figures were 2·80, 2·86, 2·83, whereas two preparations, isolated by means of aqueous extraction, gave values of 2·78 and 2·35; the viscosity of 0·90 % NaCl being taken as 1·00. After heating with 0·1 N acetic acid at 95° for 10 min., with removal of the acid by dialysis, and recovery of the product by drying *in vacuo* from the frozen state, the A-substance, dissolved in 0·90 % NaCl, showed a relative viscosity of 2·07. Material which had been treated with 2 % Na_2CO_3 at 70° for 15 min. [Meyer *et al.* 1937], and likewise recovered after dialysis, also showed a considerably decreased viscosity (η, 1·27) and treatment of the undegraded high viscosity A-substance with anhydrous formamide at 150° for 1 hr., according to the technique used by Landsteiner & Harte [1940], reduced the relative viscosity of the A-substance to 1·09, a figure only slightly higher than that for 0·90 % saline. In view of the ease with which the viscosity of the A-preparations was destroyed it was considered of interest to investigate the influence, on viscosity and serological activity, of heating the material dissolved in buffered saline at pH 3·0, 5·0, 6·9 and 8·0. The change in viscosity with time of heating an 0·5 % solution at different pH values is shown in Fig. 1. The solutions were sealed in ampoules before heating. The rapidity with which the viscosity is lost when the A-substance is heated on the alkaline side of neutrality (pH 8·0) is of interest and indicates that the A-substance should on no account be heated at alkaline reaction. It is clear that on the acid side of neutrality the A-substance is definitely more stable, although considerable loss in viscosity takes

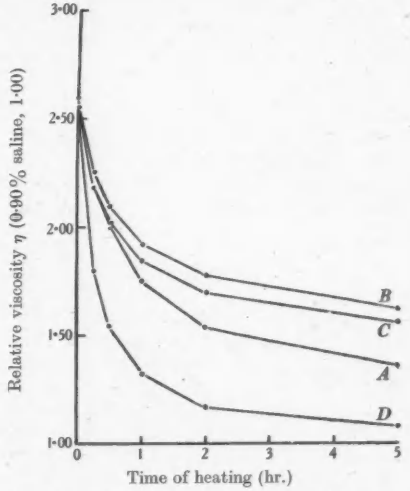

Fig. 1. Curves showing the fall in viscosity of undegraded A-substance on heating at 100° in buffered saline at pH 3·0, *A*; pH 5·0, *B*; pH 6·9, *C*; pH 8·0, *D*.

place on prolonged heating, even at pH 5·0 or 6·9. It is evident that such treatments induce a greater or lesser degree of physical degradation according to the conditions selected. The profound influence of some of these methods of treatment on the capacity of the A-substance to inhibit isoagglutination is shown in Table 5 and is discussed later.

An aqueous solution of the A-substance does not precipitate when treated with an equal volume of 2 % solutions of the following reagents: picric acid, trichloroacetic acid (10 %), aluminium sulphate, phosphotungstic acid (with or without 5 % H_2SO_4), lead acetate, ferric chloride, potash alum, Fehling's solution and silver nitrate. The addition of 0·01 M mercuric acetate, however, causes immediate gelation. Similarly, the addition of an equal volume of 0·05 M borate buffer (pH 8·5) to a 1·0 % solution of the A-substance gives rise to gel formation; the system, however, still retains pronounced elastic properties. The elastic quality may be readily shown by rotating a thin gelatinous solution in a suitable vessel and observing the return motion that follows immediately the original rotational motion has stopped. Air bubbles rising through a gel prepared in this manner from a 1 % solution assume a pear shape, the tail of each bubble remaining attached to the bottom of the container and becoming long and thin as the bubble slowly rises to the surface. The sol-gel change is reversible. Dialysis of the gel at 0° against distilled water leads to the formation of the original viscous solution; the addition of borate buffer will again give rise to a firm, elastic gel. These reversible sol-gel changes do not occur with degraded preparations of the A-substance.

Chemical composition. The A-substance gives no positive colour test when treated directly with *p*-dimethylamino-benzaldehyde but gives rise to a strong purple colour if previously heated at 100° with 0·05 N Na_2CO_3 for 6 min., according to the method of Morgan & Elson [1934]. The colour developed is equivalent to that given by a content of 12–13 % of N-acetylglucosamine. This high value was difficult to explain in view of the fact that the preparations of A-substance showed only 0·4–0·6 % of reduction by Somogyi's [1926] copper reduction method. In order to determine the N-acetylhexosamine colorimetrically, however, the A-substance must be heated with 0·05 N Na_2CO_3 and it was found that during this treatment the reducing power of the substance increased rapidly to a value between 3·0 and 3·5 % for different preparations. Heating for 6–30 min. gave rise to a maximum figure of 3·6 % reduction. The reducing power of N-acetylglucosamine, determined by the above method, is equivalent to 27 % of an equal weight of glucose; therefore, if we assume the whole of the reduction (3·3 % average value) is due to N-acetylglucosamine, then the N-acetylglucosamine colour should be equivalent to 12·2 % N-acetylglucosamine, whereas values between 12 and 13 % were found for different preparations. Several complex polysaccharide substances that were known to contain hexosamine units, such as hyaluronic acid, chondroitinsulphuric acid and the O somatic antigens of *Shigella dysenteriae* and *Eberth. typhosa*, as well as the simple methylhexosides, N-acetyl- and N-benzoylmethyl glucosamides, failed to give any colour when tested with Ehrlich's reagent after heating with 0·05 N Na_2CO_3. These results suggest that within the A-polysaccharide complex there exists an alkali-labile glycoside linkage involving the first carbon atom of the N-acetylhexosamine component. We have observed that the reddish purple colour develops with the A-polysaccharide rather more slowly than with N-acetylglucosamine; colorimetric readings should, therefore, be taken at the end of 90 min., rather than after 45 min. as originally suggested. An identical colour reaction, representing 10–11 % N-acetylglucosamine, is given by the formamide-degraded A-substance.

The immunological properties of the A-substance

A measure of the distribution of the blood group A-substance throughout the different fractions arising during the process of isolation was obtained by means of the ability of the A-factor to (1) inhibit isoagglutination and (2) prevent the haemolysis of sheep erythrocytes by an anti-A rabbit immune serum.

The isoagglutination inhibition test was carried out as follows. Diminishing concentrations of the preparation under test (0·1 ml.) were mixed with 0·1 ml. of a human anti-A (α)-serum, representing in amount 3–4 completely agglutinating doses, and allowed to stand at room temperature for 1 hr. Freshly drawn A-erythrocytes (0·5 % in 0·9 % NaCl) were then added (0·1 ml.). The mixtures were thoroughly shaken, set aside for 2 hr. at room temperature and then examined for isoagglutination. The dilution of the A-preparation allowing minimal agglutination—that is, a few groups of 2–3 cells present in an otherwise unagglutinated microscopic field—was taken as the end-point of inhibition. The sterile anti-A serum, used as a standard α-agglutinin reagent throughout the investigation, was stored without antiseptic in sealed ampoules at −20°. The serum was tested from time to time against the erythrocytes of four group A_1 persons of average sensitivity, working in the laboratory; the agglutination end-point remained constant, throughout the investigation, at a dilution of 1:192. An additional check on the inhibition test was obtained by including a 'standard' purified gastric mucin preparation in each batch of tests; the inhibition titre obtained with this material remained unchanged over a period of 2 years.

The haemolysis test was made as follows. Dilutions of the gastric mucin (0·5 ml.) were mixed with 2–3 minimal haemolytic doses (M.H.D.) of anti-A rabbit immune serum (0·5 ml.), and 0·5 ml. of 1:10 dilution of fresh guinea-pig complement was added to each tube. The mixture was incubated at 37° for 1 hr. and 0·5 ml. of a 4 % suspension

Table 5. *The inhibition of A-isoagglutination by different preparations of A-substance obtained from gastric mucin*

A-substance prepared by	Subsequent treatment	Degree of isoagglutination Dilution* of A-preparation used in test 1:10⁴	1:2 ×10⁴	1:4 ×10⁴	1:8 ×10⁴	1:1·6 ×10⁵	1:3·2 ×10⁵	1:6·4 ×10⁵	1:1·28 ×10⁶	1:2·56 ×10⁶	Control
Method 1 (water or saline extraction and Na_2SO_4 fractionation)	Untreated	0	0	0	0	0	0	1	1	2	4
	Autoclaved (121°) 15 min. at 15 lb./sq. in.	0	0	0	0	0	1	2	2	3	
	Heated to 100° for 2 hr. in aqueous solution	0	0	0	0	0	1	2	3	3	
	Fractionated between 90 and 92% acetic acid	0	0	0	0	0	0	1	2	2	
	Heated in 2% Na_2CO_3 at 70° for 15 min.	0	0	1	1	2	2	3	4	4	
Method 2 (phenol extraction)	Untreated	0	0	0	0	0	0	0	1	2	4
	Autoclaved (121°) 15 min. at 15 lb./sq. in.	0	0	0	0	0	0	1	2	2	
	Heated to 100° for 2 hr. in aqueous solution	0	0	0	0	0	1	1	2	3	
	Heated in 2% Na_2CO_3 at 70° for 15 min.	0	0	0	1	1	2	3	3	—	
	Heated in formamide, 1 hr. at 150°	0	1	2	2	3	3	3	3	—	
Material from crude gastric mucin insoluble in 90% phenol	None	1	2	4	4	4	4	4	—	—	
Protein fraction of crude gastric mucin soluble in 50% ethanol-phenol	None	4	4	4	4	4	4	4	—	—	

* Final dilution in the tubes is three times the dilution given in the table.

Table 6. *The inhibition of the haemolysis of sheep cells in* 2–3 M.H.D. *of anti-A rabbit serum by preparations of A-substance*

A-substance prepared by	Subsequent treatment	Degree of haemolysis Dilution* of A-material used in test 1:10⁵	1:10⁶	1:2 ×10⁶	1:4 ×10⁶	1:8 ×10⁶	1:16 ×10⁶	1:32 ×10⁶	1:64 ×10⁶	1:128 ×10⁶	Control
Method 1 (water or saline extraction and Na_2SO_4 fractionation)	Untreated	0	0	0	0	0	0	1	2	4	4
	Autoclaved (121°) 15 min. at 15 lb./sq. in.	0	0	0	0	0	0	1	1	2	
	Heated to 100° for 2 hr. in aqueous solution	0	0	0	0	0	0	1	2	4	
	Fractionated between 90 and 92% acetic acid	0	0	0	0	0	0	0	1	3	
	Heated in 2% Na_2CO_3 at 70° for 15 min.	0	0	0	0	0	0	1	2	3	
Method 2 (phenol extraction)	Untreated	0	0	0	0	0	0	1	2	3	4
	Autoclaved (121°) 15 min. at 15 lb./sq. in.	0	0	0	0	0	0	1	2	2	
	Heated to 100° for 2 hr. in aqueous solution	0	0	0	0	0	0	0	1	2	
	Heated in 2% Na_2CO_3 at 70° for 15 min.	0	0	0	0	0	0	1	2	3	
	Heated in formamide, 1 hr. at 150°	0	0	0	0	0	0	1	2	4	
Material from crude gastric mucin insoluble in 90% phenol	None	4	4	4	4	4	4	4	4	4	
Protein fraction of crude gastric mucin soluble in 50% ethanol-phenol	None	4	4	4	4	4	4	4	4	4	

* Final dilution in the tubes is four times the dilution given in the table.

of washed sheep red cells was added. The greatest dilution of the A-preparation allowing just visible haemolysis to occur was taken as the end-point of the haemolysis inhibition. The control tube containing saline in place of the gastric mucin dilution showed complete haemolysis in 30 min. Because of the variable nature of the haemolytic system all the results given in Table 6 were obtained in a single experiment and are therefore strictly comparable.

The results of titrations by both serological tests are given in Tables 5 and 6. The end-point of inhibition of isoagglutination given by preparation GM. 280, which was prepared by aqueous extraction of the crude mucin (method 1), is not significantly different from that given by preparation GM. 240, which was obtained by the use of 90% phenol (method 2). The first traces of isoagglutination appear in the tube containing 0·1 ml. of a $1:6{\cdot}4 \times 10^5$ (GM. 240) or $1:1{\cdot}28 \times 10^6$ (GM. 280) dilution of the A-preparation. The absolute amount of the A-substance present is about 0·1 μg. It will be observed from the results (Table 5), however, that heating these preparations in neutral saline at 100° for 120 min. causes a decrease (50–75%) in the power of the preparations to inhibit isoagglutination. Similarly, treatment with 2% Na_2CO_3 at 70° for 15 min. reduces considerably the activity of the preparation, and treatment with formamide at 150° for 1 hr. destroys more than 95% of the power of the preparation to inhibit isoagglutination. A 1% solution of the A-substance in 0·05N Na_2CO_3 shows less than 1% of the original activity (inhibition of isoagglutination) after heating at 100° for 15 min. After heating at pH 3·0, 6·9, 5·0 and 8·0 in sealed ampoules for 5 hr. at 100°, the capacity of the A-polysaccharide to inhibit isoagglutination fell to 12, 25, 25 and 2% respectively of the original value.

When the serological activity of the A-preparation is measured by the haemolysis inhibition test, however, a different picture is presented (Table 6). Again the serological activity of the undegraded material prepared by either method appears to be the same, and the absolute amount of A-substance, giving rise to almost complete inhibition of haemolysis when mixed with 2–3 M.H.D. of an anti-A rabbit serum prepared against human erythrocytes, is about 0·01 μg. Treatment with 2% Na_2CO_3 at 70° for 15 min., with formamide at 150° for 1 hr. or with neutral saline at 100° for several hours, however, causes no destruction of the capacity of the preparation to inhibit haemolysis. Indeed, it will be seen that a slight increase in activity occurs on heating the A-substance at 100° for 2 hr. An increase in activity of this order for formamide-treated material has already been reported by Landsteiner & Harte [1940]. The use of an anti-serum (rabbit) to gastric mucin (2–3 M.H.D.), in place of the anti-A erythrocyte serum gave similar results [Morgan, 1943].

A specimen of the purest preparation of A-substance was tested for antigenicity in rabbits, 0·1 or 1·0 mg. being injected intravenously at 3–4 day intervals. After three or six doses, serum samples were collected 7 days after the last dose and titrated for anti-A immune body by means of the agglutination reaction with fresh human A-erythrocytes. In no instance was any significant rise in anti-A (α)-agglutinins recorded. An account of the immunological properties of the purified A-substance is given in greater detail elsewhere. It will suffice here to state that the conversion of the A-substance into a full antigen possessing A specificity has already been accomplished [Morgan, 1941; 1943].

Attempts to remove the amino-acid-containing components from the A-substance

In some respects the chemical nature of the A-substance resembles that of the O antigens of certain gram-negative bacteria. These biologically important complexes contain a large proportion of polysaccharide, a conjugated protein component making up the remaining part of the structure essential for the manifestation of antigenicity. Earlier work [Morgan & Partridge, 1940; 1941; 1942; Partridge & Morgan, 1940] on the nature of these substances has shown that in certain instances the bacterial antigenic complex can be dissociated into its component molecules by special means, and this knowledge suggested that the A-substance should be submitted to similar procedures with the hope of attaining a complete or partial dissociation of the component molecules. The methods employed and the results obtained are briefly described.

Fractionation from phenol solution. A-substance (2·0 g.) was treated with 250 ml. of 90% phenol, allowed to stand for 24 hr. and spun for 1 hr. in a Sharples centrifuge. The thin gelatinous substance which collected on the walls of the centrifuge bowl, and which represented the portion of the A-substance that was less readily soluble in phenol, was washed with ethanol to remove phenol, and dried. It weighed 0·83 g., possessed $[\alpha]_{5461} + 13°$, contained 5·8% N and showed a relative viscosity in saline of 2·85. The dilution that showed almost complete inhibition of isoagglutination was $1:6{\cdot}4 \times 10^5$. The phenol supernatant fluid was dialyzed until free from phenol and evaporated *in vacuo* to dryness. The material recovered weighed 0·56 g., showed $[\alpha]_{5461} + 8°$, contained 5·4% N and was only slightly less active than the original material in inhibiting isoagglutination. Relative viscosity, η, 1·59.

It has been observed that dry preparations of the purified A-substance prepared by either of the methods described are never completely soluble in 90% phenol, although originally the material was readily soluble in liquid phenol of this strength and could be repeatedly dissolved in the solvent and precipitated by the addition of 10% ethanol. The fractional extraction of the purified and dry A-

substance with 90% phenol indicates that the preparation is most probably chemically homogeneous, but is certainly not homogeneous with regard to particle size. The high viscosity of the phenol-insoluble portion when dissolved in neutral saline suggests that the A-substance is present as long chain aggregates of considerable molecular size, whereas the low viscosity of the phenol soluble portion indicates that this material consists of depolymerized aggregates having smaller chain lengths in aqueous solution.

Fractionation from formamide solution. The A-preparation (3·0 g.) was dissolved in 125 ml. of anhydrous formamide, cooled to 0° and treated with an equal volume of ethanol previously cooled to −20°. No precipitate was formed up to 45% (v/v) ethanol but a considerable amount of material separated when the ethanol concentration was increased to 50%. The preparation was collected and the material remaining in the ethanol-formamide was recovered by dialysis and evaporation. The substance thrown out of solution by ethanol was again dissolved in formamide, and the precipitation repeated. The thrice precipitated material weighed 1·1 g., showed $[\alpha]_{5461}+16°$, contained 5·4% N and was active in inhibiting isoagglutination at a dilution of $1:6·4\times10^5$; η, 2·87; ash, 0·4%. The material recovered from the combined ethanol-formamide solutions weighed 1·07 g., showed $[\alpha]_{5461}+18°$; N, 5·7%; η, 1·57; and was, by both serological tests, as active as the original material.

Fractionation from aqueous solution by organic solvents. A 1·6% solution of the A-preparation was used in each experiment. Treatment with acetone gave rise to two fractions, collected over the ranges 39–41% (v/v) and 41–50% (v/v) of acetone. Practically the whole of the material was precipitated by ethanol between the levels 47–50% (v/v) and by acetic acid between 90 and 92% (v/v). In each instance, however, the precipitated products possessed the same specific rotation, N content and serological titre as the original material.

Fractionation with ammonium sulphate. A-substance (5 g.), dissolved in 300 ml. water, was fractionated by addition of solid ammonium sulphate. Up to 72% of saturation, only a small amount of material separated, and this appeared to be similar to the original preparation. The main bulk (2·74 g.) of the A-substance was precipitated on raising the ammonium sulphate concentration to 81% of saturation. The material was collected, dialyzed free from the salt, precipitated with ethanol and dried *in vacuo*. The properties, $[\alpha]_{5461}+13°$; N, 5·4%, of this fraction were substantially the same as those of the original material. Removal of the salt from the 81% saturated supernatant fluid by dialysis and recovery of the material, however, gave rise to 0·62 g. of material, which was of low serological activity as measured by the isoagglutination inhibition test. The substance showed, $[\alpha]_{5461}-14°$, and possessed a relatively low N content (4·6%). The main fraction (2·74 g.) was redissolved in water and again fractionated as above, yielding a further 0·12 g. of similar material showing *laevo* rotation and low N. A third fractionation of the main fraction gave an additional small quantity of material showing similar properties, $[\alpha]_{5461}-5°$ N, 4·3%, and possessing very low activity measured according to the isoagglutination and isohaemolysis inhibition tests. At first it was thought that these fractions, which were readily soluble in high concentrations of ammonium sulphate, and differed sharply from the original A-substance in chemical and serological properties, might represent the partly separated polysaccharide component of the A-substance. No definite evidence for this conclusion has, however, been obtained.

The above experiment yielded a highly purified sample of the A-substance. This material, thrice fractionated with ammonium sulphate, showed the following properties; $[\alpha]_{5461}+12°$; N, 5·63%; η, 2·59: maximum dilutions showing almost complete inhibition of isoagglutination, $1:6·4\times10^5$, and of haemolysis $1:32\times10^6$, respectively.

Fractionation with ammonium sulphate from alkaline solution. The technique was similar to that described above, but after each addition of the salt NaOH was added to maintain alkalinity. Only a single fractionation was performed. The results were similar to those obtained with the neutral solution of the material described above. The small fraction (5%) soluble in high concentrations of the salt showed the following properties $[\alpha]_{5461}-27°$; N, 4·6%. The activity of this material, as measured by both serological tests, showed only a few per cent of the original value.

Fractionation after heating with acetic acid. The possibility of separating the amino-acid component from the A-substance after degradation of the complex by heating with dilute acetic acid was investigated. A 1·6% solution (100 ml.) was heated at 100° with 1% acetic acid for 2 hr. The opalescence of the solution increased until it became quite opaque, but no material separated from solution. Without neutralization of the acid the solution was treated with ammonium sulphate. The first and main fraction was thrown out of solution between 70 and 85% saturation with the salt. This material separated, during dialysis, into a water-soluble and a water-insoluble part; the latter fraction, however, dissolved readily on the addition of a little Na_2CO_3. The water insoluble-material weighed 0·55 g., showed $[\alpha]_{5461}+9°$, contained 5·9% N and was as active in the inhibition of haemolysis as the original material. The water-soluble fraction showed a lower relative viscosity (η, 1·37) compared with the original unheated material (η, 2·83), the rotation was somewhat higher, $[\alpha]_{5461}+20°$, and the N content was unchanged (5·6%). This material, apart from being degraded by treatment with hot acetic acid, was essentially unchanged chemically, but the capacity to inhibit isoagglutination was only about one-quarter of the original value. Once again the small fraction of the original material soluble in high concentrations of ammonium sulphate (above 85% saturation) showed a *laevo* rotation, $[\alpha]_{5461}-20°$, a somewhat lower N (4·9%) and was only weakly active in the isoagglutination and haemolysis inhibition tests.

The action of trypsin. A purified preparation of gastric mucin A-substance (1 g.) was dissolved in 100 ml. of 15% urea solution and the pH was adjusted to about 9·0 with Na_2CO_3. Commercial trypsin (100 mg.) was then added and the mixture incubated at 37° for 6 hr. The solution was then dialyzed at 0° to remove urea, and concentrated to about 10 ml. Solid ammonium sulphate was added with thorough mixing and three fractions were removed. A small amount of material (45 mg.) separated on the addition of ammonium sulphate up to 72% of saturation. The material contained 5·5% N but was not clear enough for polarimetric examination even after the addition of alkali. The second and main fraction (0·62 g.), separating between 72 and 79% saturation, contained 5·6% N and showed $[\alpha]_{5461}+12°$. A small amount of material (60 mg. N, 4·7%) separated between 79% and full saturation at 18°, and a

further 65 mg. of material was finally recovered from the saturated ammonium sulphate solution. All fractions, with the exception of the last (not tested), showed no very significant change in serological activity. The treated material, however, had a lower viscosity (2·17) than the original preparation.

Treatment with chloroform-amyl alcohol. A 2% solution of A-substance ($[\alpha]_{5461}+12°$; N, 5·70%) was shaken with an equal volume of $CHCl_3$-amyl alcohol mixture according to the method of Sevag [1934]. The aqueous layer, after several treatments with the $CHCl_3$-amyl alcohol mixture, was fractionated with ammonium sulphate. The main fraction, 63% of the original material, separated between 73 and 82% of salt, showed a specific rotation $[\alpha]_{5461}+12°$ and contained 5·25% N. An additional 10% was recovered from the supernatant fluid. This fraction showed a lower N content but was otherwise similar to the main fraction. Both materials gave inhibition of isoagglutination at a dilution of $1:6{\cdot}4\times10^5$.

It would appear that the application of the techniques described have in no instance succeeded in removing the amino-acid components from the polysaccharide moiety of the A-substance. The application of certain of these procedures, however, removes about 5% of material which shows a *laevo* rotation and possesses a lower N and a considerably diminished specific activity when compared with the purified A-substance.

Acid hydrolysis of the A-substance

The A-substance is readily hydrolyzed by heating in N HCl at 100°. Complete liberation of the component amino-acids, however, is only attained after hydrolysis with 6N HCl. The hydrolysis was carried out in sealed ampoules, samples being removed from time to time and immediately neutralized. during hydrolysis was followed by Van Slyke's method [1929] and it is to be noted that any free amino-groups liberated through the elimination of the acetyl groups from N-acetylhexosamine are included in the results. The amino-acids set free during the hydrolysis were estimated by the method of Van Slyke & Dillon [1938] as modified by Van Slyke, Dillon, MacFadyen & Hamilton [1941] and the results are expressed as 'carboxyl-N'. Glucosamine is not determined under these conditions. 'Hexosamine' was determined according to the method of Elson & Morgan [1933] and is expressed as glucosamine. N-acetylglucosamine was found to be converted almost completely to glucosamine by heating with N HCl at 100° for 2 hr. Reducing substances reach a maximum value after about 4 hr. heating, whereas the optical rotation, the 'hexosamine' and the amino-N reach maximum values between 6 and 12 hr. hydrolysis. The maximum figure for amino-acid is reached only after several hours' heating with 6N HCl.

The maximum figures for amino-acid N (2·2%) and for hexosamine-N (2·3%) are together approximately equal to the maximum amino-N value (4·5%). During the early stages of the hydrolysis, however, the total amino-N value (Van Slyke's method) is greater than the sum of the amino-acid and hexosamine figures. This is most probably due to the fact that the Van Slyke method assays the terminal NH_2 groups of polypeptides, whilst the amino-acid determination (ninhydrin method) is specific for free amino-acids only. There remains a considerable discrepancy between the maximum amino-N value (4·5%) and the total N of the preparation (5·7%). If, however, all the N (3·5%) not

Table 7. *The hydrolysis of A-substance by* HCl *at* 100°

Acid strength	Time of heating (hr.)	$[\alpha]_{5461}$ ($c=1\%$, $l=1$)	Reducing power (glucose =100)	Hexosamine* (%)	Nitrogen (%)			
					Total	α-Amino	Amino-acid	Hexosamine
N	0	+0·11°	0·5	0·0	5·72	0·19	0·0	0·0
	1	+0·35°	45	24·3	—	3·14	0·19	1·90
	6	+0·52°	50	26·9	—	4·02	0·46	2·05
	12	+0·51°	45	27·3	—	4·20	0·80	2·17
	24	+0·50°	—	26·6	—	4·29	1·32	2·24
6N	1	+0·44°	42	27·0	—	4·27	0·99	2·19
	6	+0·32°	43	24·0	—	4·55	2·17	1·88
	12	+0·24°	—	—	—	3·66	1·75	—

* Estimated by the colorimetric method.

The analytical figures obtained during the course of the hydrolysis of a typical preparation are given in Table 7. The figures largely confirm the results obtained earlier by Landsteiner & Harte [1940]. The reducing power was determined by Somogyi's modification [1926] of the Shaffer & Hartman method [1921] and the results are expressed in terms of glucose. The liberation of α-amino groups accounted for as hexosamine is assumed to be in the amino-acid component, it follows that the latter comprises about $3{\cdot}5\times6{\cdot}4$ or 22% of the total complex. The maximum figure obtained for reducing substances is 52%, calculated as glucose. Differentiation of the reducing substances into fermentable and non-fermentable components by the rapid yeast 'absorption' method of Harding & Selby [1931]

revealed that the fermentable sugar did not exceed 3–4 % of the maximum reducing substances at any stage of the hydrolysis. If the hexosamine present is considered as glucosamine, which has quantitatively the same reducing power as glucose under the conditions of estimation employed, then the hexosamine accounts for 27 % of the reduction. If the remaining reduction (25 %) is assumed to be due to galactose, then this sugar is present to the extent of 32 %, since its reducing power, relative to glucose, is only 72 %. Qualitative tests for the presence of individual amino-acids show that tyrosine and cysteine are absent or present in very small amounts.

DISCUSSION

The isolation of the specific A-polysaccharide substance from crude gastric mucin by methods that avoid the use of mineral acid or of alkali, and enable the isolation procedures to be carried out at normal temperatures, is described.

The methods give an excellent yield of a serologically active polysaccharide-amino-acid complex, which possesses in full the property shown by crude gastric mucin of inhibiting the isoagglutination of A-erythrocytes by natural anti-A (α)-agglutinin (human), retains completely the high viscosity of the original mucin, and is essentially homogeneous when examined at pH 4·0 or 8·0 in the Tiselius electrophoresis apparatus. The material can be considered as the blood group A-substance largely in its undegraded state and it is noteworthy that these two radically different methods for the isolation of the active substance yield apparently identical products.

The A-substance prepared by either method is similar in composition to that described by Landsteiner & Harte [1940]. There are, however, a number of important differences in the physical and serological properties of our preparations of A-substance and those described by other workers. Preparations of A-substance made by either of the methods described in this paper show not only high serological activity, as determined by the inhibition of isoagglutination, but also considerable viscosity, and the property of forming an elastic gel on the addition of borate buffer at pH 8·5. The latter property is readily lost and it has been found that even slightly degraded preparations of the A-substance fail to form an elastic gel on the addition of the borate buffer. Thus, the addition of this reagent to a solution of A-substance will reveal at once whether the material has been degraded during the course of its preparation. In addition to the rapid decrease in the viscosity of the A-substance that arises from heating solutions of the substance, there is a steady fall in the capacity of the preparation to inhibit the isoagglutination of A-cells by either natural human anti-A (α)-agglutinin or by the anti-A agglutinin of rabbit immune serum. The insensitive nature of the agglutination reaction as usually carried out, with progressively doubled dilutions of the A-substance, does not allow small changes in the capacity of an A-preparation to inhibit isoagglutination to be observed. For this reason it is not possible to detect a decrease in the power of a preparation to inhibit isoagglutination when the destruction is much less than 50 % of the original value. Nevertheless, it is apparent that heating at 100° or simple sterilization at 120° in neutral solution causes a loss in isoagglutination inhibiting activity, whereas almost complete destruction of this particular serological activity is brought about by heating with formamide or warming with dilute sodium carbonate. Although exposure to these conditions leads to drastic loss in viscosity and almost complete destruction of the power to inhibit isoagglutination, it is of interest to find that the resulting substance in each instance shows unchanged the property of inhibiting the haemolysis of sheep cells by anti-A rabbit immune serum. Indeed, there is evidence that heating with formamide may even increase this particular activity, as was originally observed by Landsteiner & Harte [1940]. These workers stated that the increase in anti-lytic activity is most probably due to a structural change, since 95 % of the starting material with about twice the potency could be recovered after this treatment. Jorpes & Norlin [1933; 1934] and Jorpes [1934] reported that the action of trypsin on the blood group A factor in human urine considerably decreased the anti-agglutinating activity but that the anti-lytic activity was not destroyed. They found that anti-agglutinating and anti-lytic factors could be separated by precipitating the anti-agglutinating factor from solution by tannin. Jorpes & Norlin consider that the anti-agglutinating factor is in this instance protein in nature, the anti-lytic substance being essentially polysaccharide. With material of high anti-lytic activity, we have not so far detected any chemical difference between preparations of the A-substance that show high or low activity in inhibiting isoagglutination and Landsteiner & Harte [1940] reported that they were unable to effect a separation of the A-substance into material showing different chemical and serological properties. Degradation of the native A-substance under the conditions devised by earlier workers for its isolation and purification almost certainly accounts for the low viscosity and much decreased power to inhibit isoagglutination, both of which are recorded in the literature for purified preparations, whereas the stability of the haemolysin-inhibiting property, when exposed to similar conditions, offers an explanation for the preservation of the intense anti-

haemolytic activity of these preparations. It must be emphasized that up to the present the preparations of A-substance, used by different workers, have been obtained almost exclusively from one source. This material, Wilson's gastric mucin (Type 1701-W), is the one most commonly used by bacteriologists for enhancing the virulence of bacterial cultures. We have examined one specimen of gastric mucin obtained in this country from Light and Co. and have found it to be very similar to the Wilson product. It is not known to what extent the A-factor in these commercial materials is degraded during their preparation. Our observations on the ease with which certain properties of the A-substance are destroyed suggest that further investigation of the properties of native gastric mucin should be undertaken with respect to its power to inhibit isoagglutination and A specific haemolysis and the relation of these activities to viscosity and power to increase the virulence of bacterial cultures. It is of interest that we have isolated from the fluid of pseudo-mucinous ovarian cysts a polysaccharide complex that is two or three times as active in inhibiting A-isoagglutination as the most active material isolated from hog gastric mucin. This material is, however, less viscous than hog gastric mucin A-substance.

It is not possible to compare accurately the activity of our preparations with those of other workers, because in many instances little or no indication is given of the strength of the anti-A (α)-agglutinin or of the anti-A rabbit haemolysin used in the tests. By replacing our test dose of anti-A (α)-serum by a quantity of serum only just sufficient to give complete agglutination of the A-cells used in the test, we found the amount of A-substance giving rise to a significant although incomplete inhibition of isoagglutination to be about 0·01 μg. Similarly, haemolysis-inhibition tests in which 1 M.H.D. only of the lytic agent was employed showed that as little as 0·001 μg. of the undegraded A-substance could be detected. Smaller absolute values which are, however, of doubtful value, can readily be obtained if the same pipette is used throughout to make the serial dilutions of the A-substance.

The question arises as to why the A-substance, which contains amino-acids as an integral part of the molecular complex and is superficially analogous to the O antigens of certain bacteria, is not itself antigenic. Closer examination shows, however, that the A-substance is not truly analogous to these bacterial antigens, which appear to be large polymolecular complexes built by the combination of polysaccharide and protein molecules through secondary valency bonds. Some of these biologically important aggregates can be dissociated into their component molecules with complete loss of the original specific antigenic property. The A-substance, however, is not separable into its component molecules by these procedures and it would appear, therefore, that the amino-acid components are in this instance more firmly linked, most probably by normal primary valency bonds, to the carbohydrate units of the polysaccharide molecules. Furthermore, it seems probable that the amino-acids are present either as single molecules or as short polypeptide chains and that the A-substance does not in fact contain an intact protein component. In support of this suggestion it has been shown that the A-substance can be converted into a full antigen that gives rise to specific A immune-body, by combination with a suitable protein molecule. The conditions employed to synthesize this functional polymolecular aggregate suggest that the component molecules—A-substance and conjugated protein—are held together by secondary valency bonds only. An additional factor, however, that may well be responsible for the non-antigenicity of the A-substance, is its lack of tyrosine. The absence of this amino-acid has generally been considered to render a protein devoid of antigenic properties, although some evidence against this view has recently been given.

The preparation of the purified A-substance in large amounts has permitted an investigation into the nature of the constituent in commercial gastric mucin that is responsible for its 'virulence enhancing' power, a property which bacteriologists and immunologists have put to much practical use during the last few years. The undegraded A-substance appears to possess 'virulence enhancing' properties, whereas all other substances in gastric mucin, including the phenol-soluble protein component, are inactive in this respect. Any treatment, however, such as sterilization by heat, which destroys the viscosity of the A-substance and reduces the capacity of the preparation to inhibit isoagglutination (even when the power of the resulting material to inhibit the haemolysis of sheep cells by anti-A serum is completely retained) leads to destruction of the 'virulence enhancing' property.

SUMMARY

1. Two methods are described for the isolation from crude gastric mucin of a polysaccharide-amino-acid complex showing intense blood group A specificity.

2. The undegraded A-substance, which is essentially homogeneous, gives rise to slightly opalescent 1 % aqueous solutions that show considerable viscosity. The substance inhibits, in extremely small amounts, specific isoagglutination and haemolysis of sheep cells by specific anti-A rabbit immune serum.

3. The native A-substance is readily degraded by heat treatment and gives rise to a substance which shows a lower viscosity in aqueous solution and which possesses a reduced power to inhibit isoagglutination. The partially degraded A-substance, however, shows a slightly increased activity in inhibiting A specific haemolysis.

4. The relationship of the purified undegraded A-substance to the 'virulence enhancing' agent in crude gastric mucin is discussed.

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ADDENDUM

Electrophoretic Examination of A-substance from Gastric Mucin

By R. A. KEKWICK

Three preparations of A-substance from gastric mucin (210, 240, 321) were examined in the Tiselius U-tube at 0° with the diagonal schlieren system for optical observation. Samples of the dried preparations were dissolved in buffers to give 2% solutions and dialyzed in cellophane membranes at 2° against several changes of the same buffer. Buffers used were acetate, pH 4·0, $\mu = 0{\cdot}1$, phosphate, pH 8·0, $\mu = 0{\cdot}1$ and phosphate-NaCl buffer, pH 8·0 (phosphate $\mu = 0{\cdot}1$ + NaCl $\mu = 0{\cdot}15$). In all these buffers very viscous solutions were obtained, which gave a considerable light scattering.

In all samples, both at pH 8·0 and pH 4·0, a single component accounted for 90% of the total refraction, the remaining 10% being associated with a somewhat ill-defined contaminant.

In pH 8·0 phosphate buffer $\mu = 0{\cdot}1$, the migration was anodic, $\mu = 0{\cdot}4 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ V.$^{-1}$, and in pH 4·0 acetate buffer $\mu = 0{\cdot}1$ a very slight cathodic migration occurred. Under each of these conditions the descending boundary remained sharper than the ascending, the reverse of what is usually observed, and this presumably was due to the high viscosity of the solution.